

PREALBUMIN

Metabolic and Chemical Studies

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Title and subtitle Prealbumin: Metabolism and Chemical Studies		
Abstract Prealbumin (PA) is a carrier protein for thyroxine and for retinol-binding protein (RBP), the direct carrier of vitamin A. Using isolated rat hepatocytes PA and RBP in plasma were shown to be of mainly hepatic origin and normally to be secreted from the hepatocytes in a molar ratio of about 1:1. In hepatocytes from rats with an acute, croton oil induced inflammatory reaction, the PA secretion rate was found to be significantly more decreased than the RBP secretion rate. Turnover experiments with ¹³¹I-labelled prealbumin showed that the fractional catabolic rate of PA was normal during inflammation and fasting in the rat. The decreased plasma concentrations of PA in these conditions were due to decreased synthesis (inflammation and fasting) and decreased intravascular fraction of PA (early inflammation). In patients with chronic inflammation the plasma concentration of free (non PA-bound) holo RBP (RBP with bound retinol) was found to be normal in spite of the decreased total plasma concentrations of PA, RBP and retinol. The plasma concentration of free holo RBP discriminated better than the mentioned total concentrations between patients with abnormal and normal dark adaptation (calculated from data in the literature). It was made probable that the rate of RBP secretion is not decreased in chronic inflammation, and that mainly the rate of PA synthesis governs the total plasma concentrations of PA, RBP and retinol. PA contains four cysteinyl residues. Using electrofocusing under denaturing conditions more than 90 per cent of cysteinyl residues of PA in fresh plasma and CSF were shown to be highly reactive in thiol-disulphide exchange reactions. Both in vitro and in vivo, reactions proceed by which the cysteinyl residues receive an additional negative charge and become unreactive. In vitro, these reactions proceeded faster in CSF than in plasma which explains the previously recognized higher negative charge and higher electrophoretic mobility of PA in CSF. The chemistry of these reactions was not revealed in detail, but it seemed as if oxidation without formation of disulphides was involved. These reactions might explain the known unreactivity of the cysteinyl residues in purified PA.		
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PREALBUMIN

Metabolic and Chemical Studies

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PETER FELDING

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AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds
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PREALBUMIN

Metabolic and Chemical Studies

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University of Lund, Malmö, Sweden

1984

This thesis is based on the following papers:

- I. Felding P, Fex G. (1983) Factors responsible for the decreased plasma concentration of prealbumin during acute inflammation and fasting in the rat. *Acta Physiol. Scand.* 117, 377-383.
- II. Felding P, Fex G. (1982) Cellular origin of prealbumin in the rat. *Biochim. Biophys. Acta* 716, 446-449.
- III. Felding P, Fex G. (1984) Rates of synthesis of prealbumin and retinol-binding protein during acute inflammation in the rat. (Manuscript)
- IV. Fex G, Felding P. (1984) Factors affecting the concentration of free holo retinol-binding protein in human plasma. *Eur. J. Clin. Invest.* 14, 146-149.
- V. Felding P, Fex G. (1984) The increased negative charge of prealbumin in cerebrospinal fluid is acquired in_vitro by oxidation of the cysteinyl residues without formation of disulphides. *Scand. J. Clin. Lab. Invest.* (Accepted)

In the text the papers will be referred to by their Roman numerals.

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INTRODUCTION

Historical

Prealbumin (PA) is a plasma protein, whose name refers to its position in front of albumin after electrophoresis. A protein with this electrophoretic mobility was early described in normal cerebrospinal fluid (CSF) (Kabat et al., 1942), and was thought to be specific for CSF since it was not at first recognized in plasma (Fisk et al., 1951). In 1955 Gabar et al., using immunoelectrophoresis, demonstrated that antiserum to human serum reacted with this component of CSF indicating that it was also present in plasma (Schultze et al., 1956). After electrophoresis in barbital buffer, pH 8.6, of serum and CSF containing ^{131}I -thyroxine, radioactivity was observed in the PA position (Robbins & Rall 1955, 1957). When encountered in plasma, it was at first believed to be due to impurities of the ^{131}I -thyroxine preparation, until Ingbar - using electrophoresis in tris-maleate buffer, pH 8.6, instead of barbital buffer - clearly demonstrated the thyroxine-binding property of PA in plasma (Ingbar 1958); and indeed, PA is still often referred to as thyroxine-binding prealbumin. A new field of research was opened when in the laboratory of DeW. S. Goodman PA was shown to be a carrier protein for the smaller retinol-binding protein (RBP), the direct carrier of retinol (vitamin A) in plasma (Kanai et al., 1968). In the years that followed, it was mainly Goodman and his colleagues, and a Swedish group around P.A. Peterson and L. Rask, who uncovered the major features of PA and of the retinol transport in plasma. The amino acid sequence of PA was reported in 1974 (Kanda et al., 1974), as was its detailed crystallographic structure (Blake et al., 1974, 1978). "Prealbumin", with similar structure and functions but varying electrophoretic mobility, has been found in all mammals and birds investigated but not in fish. To emphasize the known functions of PA and to avoid confusion with precursors to albumin, the name transthyretin was approved by the NC-IUB and the JCBN (J. Biol. Chem. 256: 12-14, 1981), though the term is rarely used for PA.

Purification

The purification of PA from serum or plasma has not posed a major problem. Its plasma concentration is not very low (about 0.3 g/l) and its unique electrophoretic position (in human) has been used both for identification and for purification purposes. Initially, PA was obtained from serum by differential precipitation of the proteins, followed by preparative electrophoresis

(Schultze et al., 1956, Got & Bourrillon 1963). Later, ion exchange chromatography (on DEAE-gels) was used as the first step (Oppenheimer et al., 1965a, Raz & Goodman, 1969). The association between PA and RBP at physiological or higher ionic strengths and the dissociation at low ionic strength were first used in the preparation of RBP from serum (Vahlquist et al., 1971), and later in the preparation of PA (Fex et al., 1977, Navab et al., 1977a). Thiol-disulphide exchange chromatography, combined with affinity chromatography on human RBP coupled to Sepharose (Fex et al., 1977), has been used in our laboratory as an easy method of isolating PA in various animal species.

Chromatography on matrix-bound thyroxine has been used in the purification of thyroxine-binding globulin (Gershengorn et al., 1977), but has not to my knowledge been used successfully in the purification of PA.

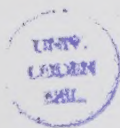
Primary structure

PA is composed of four identical subunits. The whole amino acid sequence of the subunit (Kanda et al., 1974) comprises 127 amino acids including 1 cysteinyl in position 10 from the N-terminus, 1 methionine, 2 tryptophan and 5 tyrosine residues.

PA does not contain carbohydrate (Fex et al., 1977, Peterson 1971a) and, according to the amino acid sequence, the molecular weight of the subunit is 13745. Compared to albumin, PA has a relatively high content of tryptophan and tyrosine, which explains the name "tryptophan rich prealbumin" occasionally used, and the relatively high absorbence coefficient of 280 nm ($A_{1\text{ cm}}^{1\%} = 14.1$) (Raz & Goodman 1969). These properties are, however, not unique compared to other proteins such as RBP (Rask et al., 1980). An amino acid sequence homology to the glucagon family of hormones and to RBP has been asserted, though the relationship with RBP is probably open to question (Jörnvall et al., 1981).

Higher structure

Combined with the amino acid sequence described above, X-ray analysis of the PA crystal (Blake et al., 1974, 1977, 1978, Blake 1981) gives a detailed picture of the secondary, tertiary and quaternary structure of PA, including the binding sites for the thyroid hormones but not those for RBP. This picture is in good agreement with information obtained with other methods, with the possible exception of the apparent discrepancy between the binding with high affinity of only one molecule thyroxine per molecule PA (Robbins et al., 1978), and the X-ray demonstration of two identical thyroxine binding sites per PA molecule.



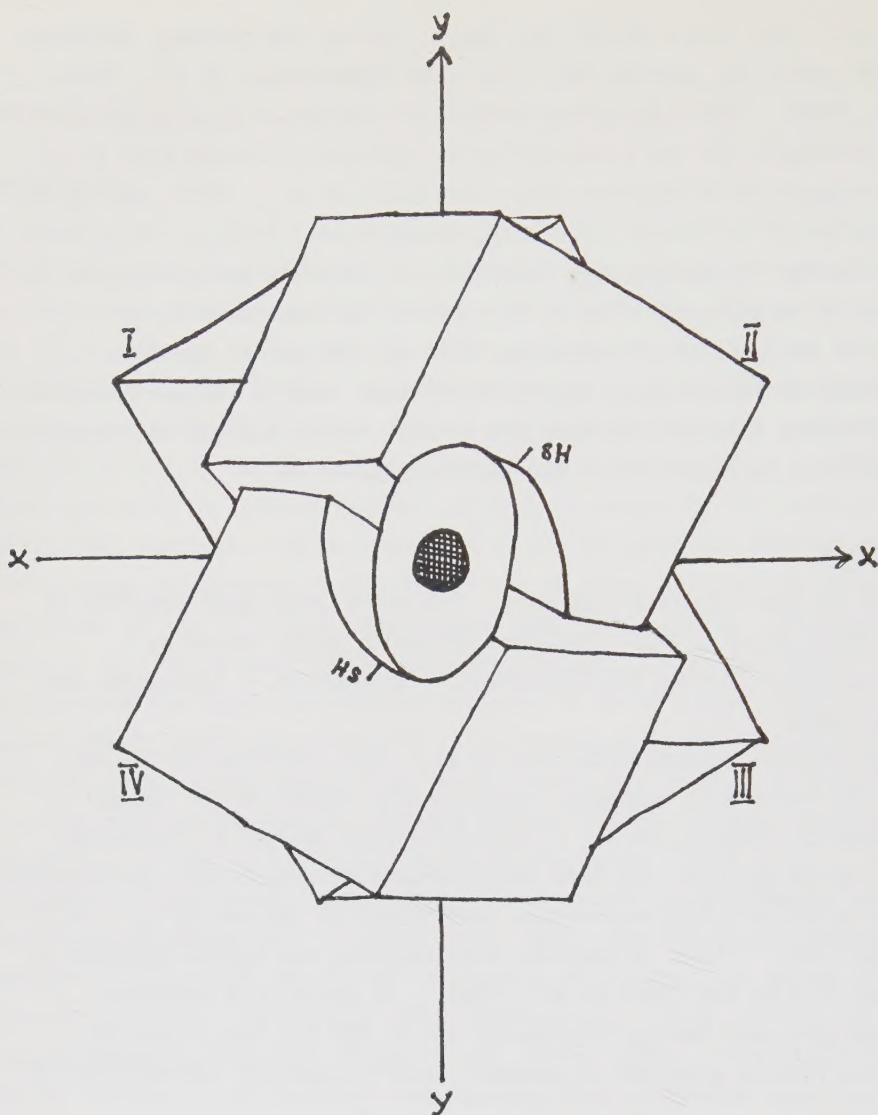


Fig.1. A schematic drawing of the prealbumin tetramer looking down the z-axis. x, y, and z are the three two fold symmetry axis perpendicular to each other. Subunit I and II constitute one of the dimers, and subunit III and IV constitute the other dimer. The central channel around the z-axis contains the two thyroxine-binding sites. The cylindrical-helical impression (the proposed DNA-binding site) runs parallel to the x-axis in the x-y plane at the positions where the y-axis enters and leaves the molecule. (The impression is hidden behind subunit II (top) and IV (bottom)).

About half the amino acids in the subunits are organized into two β -sheets with interactions face to face, each sheet consisting of four β -strands with antiparallel interactions. Further antiparallel interactions, mainly by hydrogen bonds between β -strands at the edges of the β -sheets, link two monomers into a dimer. The dimers are assembled face to face by both hydrophilic and hydrophobic interactions involving all subunits, and leaving a central channel through the molecule between the dimers. There are no covalent bonds between the subunits. The tetramer has a 222 symmetry - i.e., rotation 180° around each of three symmetry axes perpendicular to each other results in a molecular appearance indistinguishable from that of the start position. There is no plane of symmetry, and the channel axis is one of the symmetry axes (Z-axis, Fig. 1).

The overall size of the tetramer is 7.0 nm x 5.5 nm x 5.0 nm and the central channel is 5 nm long and 0.8 nm in diameter with a central constriction. It contains two identical, well-separated thyroxine-binding sites (the overall length of the thyroxine molecule is 1.2 nm). The external surface of PA has a cylindrical-helical depression at the site of the Y-axis of symmetry (Fig. 1) which is complementary to a DNA double helix. The 9 N-terminal amino acids are not organized in the structure. The single cysteine in position 10 is situated at the mouth of the central channel in the edge of the β -sheet which starts with proline in position 11.

The tetrameric structure is extremely stable, and human PA resists 0.1 % SDS as well as 8 mol/l urea, dissociating only very slowly in 6 mol/l GU HCl at neutral pH (Branch et al., 1972). It also resists changes in pH ranging from 3.5 to 12 (Branch et al., 1971). Despite this stability the tetramers do exchange subunits, although very slowly under non-denaturing conditions (Bernstein et al., 1970, Fex & Hansson, 1980). The binding of thyroxine increases the stability of the tetramer (Nilsson et al., 1975), and reduces the subunit exchange rate (Bernstein et al., 1970).

Interaction with thyroxine

Trace amounts of radio-labelled thyroxine are bound in serum to thyroxine-binding globulin (TBG), PA and albumin. Using agarose gel electrophoresis in tris-maleate buffer, pH 8.6, I found the distribution of such radioactivity in pooled serum to be 60 %, 27 % and 13 %, respectively (Felding unpublished), and these values agree with those found in other studies (Oppenheimer & Werner 1966, Moses et al., 1982). The corresponding values in concentrated pooled CSF were TBG (15 %), PA (79 %) and albumin (6 %), indicating that PA is the major carrier of thyroxine in CSF. In both CSF and serum, less than a few per cent of the PA molecules carried thyroxine. The given distributions of thyroxine

were extremely close to those calculated from the concentrations of the proteins and thyroxine and the association constants given in Table I.

X-ray analysis of PA (Blake et al., 1974, 1977, 1978, Blake 1981) shows two identical thyroxine-binding sites, one in each half of the central channel. The 4'-hydroxyl group of the thyroxine is directed towards the centre of the PA molecule. The I-atoms and other structures of the hormone can be perfectly matched with hydrophilic and hydrophobic structures inside the channel. From the crystallographic structure it seems possible to explain the different binding affinities to PA of the thyroid hormones and several analogues (Andrea et al., 1978).

General agreement exists concerning the presence of one high affinity site in PA for thyroxine, with an association constant in the range 10^7 - 10^8 mol⁻¹ obtained by different fluorescence quenching techniques (Nilson & Peterson 1971, Cheng et al., 1977), and by equilibrium dialysis (Ferguson et al., 1975, Andrea et al., 1980). The second thyroxine molecule binds with lower affinity; because of the identical binding sites this is due to negative cooperativity (Ferguson et al., 1975, Robbins et al., 1978). Steric interactions between the two thyroxine molecules would seem to be excluded, owing to their well-separated positions in the central channel (Blake 1981). Electrostatic repulsion between opposed ionized phenolic hydroxyl of the two thyroxine molecules has been made probable in experiments with thyroxine analogues (Cheng et al., 1977), but a small conformational change has also been shown to be caused in PA by the binding of thyroxine (Irace & Edelhoch 1978), and might explain the decreased affinity for the second thyroxine molecule.

Table I illustrates the effects of molecular changes between thyroxine analogues on the affinity for PA and other known thyroid-hormone-binding proteins. Only for PA the detailed structure of the binding site is known.

The difference in number or position of the iodine atoms between T₄, T₃ and rT₃ changes the affinity for PA and TBG, and changes it to a corresponding extent, indicating similarities in the corresponding parts of the binding sites. However, changes in the aliphatic side chain (T₄Ac and D-T₄ compared to T₄) affect the binding to TBG and PA differently. The binding site in the rat liver nucleus discriminates strongly between T₄, T₃ and rT₃, as can be seen in Table I; it prefers T₃, which may explain the higher biological activity of T₃. Several substances commonly used in protein chemistry, and apparently without any similarity to thyroxine, compete with thyroxine for the binding site in the central channel of PA (Blake et al., 1974). Examples of such substances are iodoacetic acid, dithiothreitol and 5,5'-diethyl barbituric acid. Of more clinical interest is the binding of the salicylate ion.

Table I

Association constants for the binding of one molecule of L-thyroxine (T_4), 3,5,3'-triiodo-L-thyronine (T_3), 3,3',5'-triiodo-L-thyronine (reverse T_3 , rT_3), tetraiodothyroacetic acid (T_4Ac) and D-thyroxine ($D-T_4$) to thyroid-hormone-binding proteins at neutral or slightly alkaline pH. The values without given units are percentages of the association constants for L-thyroxine.

	PA	TBG	Albumin	Liver nuclei
T_4	$2 \times 10^8 \text{ mol}^{-1}$	$2 \times 10^{10} \text{ mol}^{-1}$	$2 \times 10^6 \text{ mol}^{-1}$	$0.8 \times 10^8 \text{ mol}^{-1}$
T_4	100	100	100	100
T_3	8, 9.2*	9	55	800
rT_3	33*	38	100	0
T_4Ac	160, 676*	1.7	110	
$D-T_4$	3.7*	54	100	

* Values from Andrea et al., 1980; other values from the review by Robbins et al., 1978.

In contrast to TBG, PA seems to be essential to human life, since a total absence of PA has not been found to occur, but whether the thyroxine binding property is of vital importance is not known. The binding of PA to RBP is independent of the binding to thyroxine (Raz & Goodman 1969). No hard evidence has yet been reported to support any physiological significance of the combination of the two binding functions.

Interaction with retinol-binding protein

In plasma, retinol circulates in a 1:1 molar complex with RBP (Kanai et al., 1968). This complex, holo RBP, forms a 1:1 molar complex with PA under physiological conditions in plasma (Kanai et al., 1968, Rask et al., 1980). Owing to the symmetry of PA, the number of binding sites for RBP ought to be 2 or 4. The positions of the binding sites have not been found, and the maximal molar ratio RBP:PA in the complex has been said to be from 1:1 to 4:1 (Fex et al., 1979, Heller & Horwitz 1974, Kopelman et al., 1976, Trägårdh et al., 1980, Van Jaarsveld et al., 1973a). The association constant in physiological buffer for the 1:1 molar complex of PA and holo RBP has been calculated from changes of fluorescence, and by other means. Examples of published values are 1.2×10^6

1 mol^{-1} (Van Jaarsveld et al., 1973a), $7.8 \times 10^6 \text{ l mol}^{-1}$ (Trägårdh et al., 1980), $1.3 \times 10^7 \text{ l mol}^{-1}$ (Fex et al., 1979), $1.6 \times 10^7 \text{ l mol}^{-1}$ (Raz & Goodman 1969) and $2 \times 10^7 \text{ l mol}^{-1}$ (Peterson & Rask 1971). A similar binding affinity has been found in the rat (Peterson et al., 1973). The binding of PA to holo RBP makes it more difficult to extract retinol using organic solvents (Peterson 1971b), and enhances the fluorescence of retinol, indicating altered environment of the retinol molecule (Peterson & Rask 1971). Although conflicting evidence exists, the presence of retinol seems to stabilize the complex between PA and RBP (Robbins et al., 1978, Rask et al., 1980). The association constant of apo RBP from urine to PA has been estimated to be about 20-25 per cent of the corresponding constant for the same RBP after saturation with retinol (Fex et al., 1979), but the mechanism responsible for this retinol effect is at present unknown. The binding of RBP to PA is only slightly pH-dependent with maximal affinity at physiological pH (Peterson 1971b, Van Jaarsveld et al., 1973a). The complex dissociates at low ionic strength (Peterson 1971a,b), most probably due to conformational changes in RBP (Rask et al., 1972). Moreover, antibodies to RBP (but not to PA) dissociate the complex (Peterson 1971b), and iodination of RBP (but not of PA) decreases the binding affinity between the proteins (Raz et al., 1970). Thus, RBP is the more vulnerable of the proteins as far as binding properties are concerned.

The vitamin A transport

As reviewed (Glover 1983, Goodman 1976, Rask et al., 1980), vitamin A is transported from the intestines as retinyl esters in the chylomicrons to the hepatocytes where it is stored, mainly in ester form. It is then converted to alcohol, bound to RBP and secreted as holo RBP (the 1:1 molar complex of retinol and RBP) (Muto et al., 1972). In plasma, holo RBP (m.w. 21000) binds to PA (m.w. 55000) and is thereby preserved from glomerular filtration. The uptake of retinol into the target cells is believed to be mediated by holo RBP and its receptor on the cell surface (Heller 1975, Rask & Peterson 1976, Bhat & Cama 1979b). After delivery of retinol at the cell membrane, RBP loses some of its affinity, both for the receptor (Heller 1975, Bhat & Cama 1979b), and for PA (as described in the previous section), and is therefore eliminated more rapidly than holo RBP by glomerular filtration (Vahlquist et al., 1973). From the cell membrane, retinol is transferred to an intracellular retinol-binding protein different from that in plasma (Glover 1983). The above model for vitamin A transport is illustrated in Fig. 2.

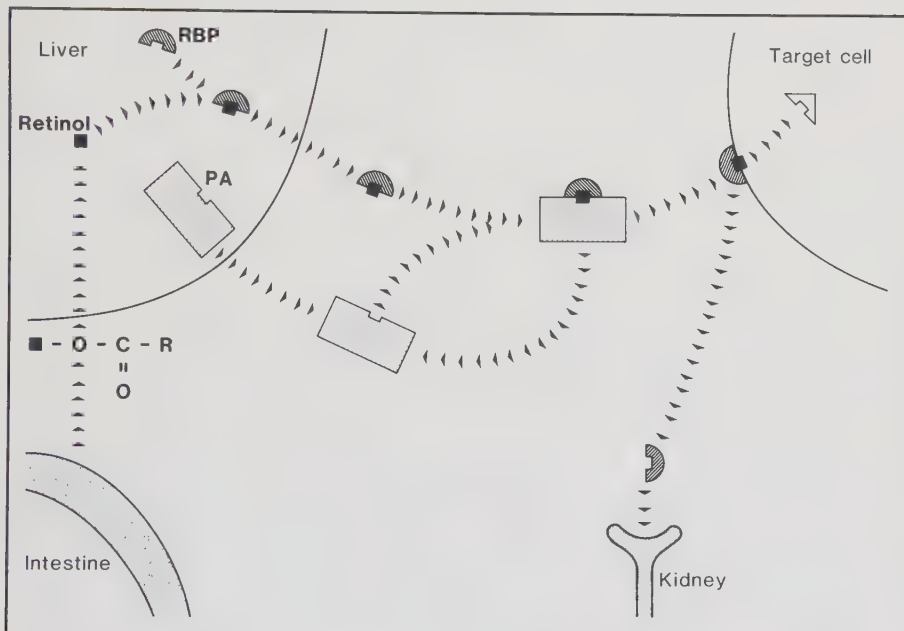


Fig. 2. The vitamin A (retinol) transport. R is an aliphatic chain connected to chylomicrons. PA is prealbumin and RBP is retinol-binding protein.

The major part of PA in plasma originates from synthesis in the hepatocytes (II, Navab et al., 1977b, Gitlin & Gitlin 1975). As for many other plasma proteins, the site of degradation is unknown. In contrast to RBP, the kidneys are not involved in the catabolism (Vahlquist et al., 1973, Smith & Goodman 1971). The amount of intravascular PA is about half of the total circulating pool and the biological half-life ($T_{1/2}$) has been found to range from 1.7 to 3.6 days in healthy humans (Vahlquist et al., 1973, Socolow et al., 1965, Oppenheimer et al., 1965b), from 10.6 to 29 h in rats (I, Peterson et al., 1974, Dickson et al., 1982), and is about 22 h in monkeys (Vahlquist 1972). In both rats, monkeys and man (III, Peterson et al., 1974, Vahlquist 1972, Vahlquist et al., 1973), PA and RBP are normally synthesized in a molar ratio of about 1:1. The molar plasma concentration ratio PA:RBP is higher, about 2:1, because of the faster turnover of RBP (refs as above).

The normal plasma concentration of PA in adult humans is about 0.3 g/l. Women have slightly lower concentrations than men, and young and old persons have lower concentrations than middle-aged (Jouquan et al., 1983). The concentration is slightly decreased in pregnancy and low in newborn infants and in children (Ganrot 1972, Gitlin & Gitlin 1975, Vahlquist et al., 1975). The effect on the PA plasma concentration of oral contraceptives is slight and varies with the formulation (Vahlquist et al., 1979). Anabolic steroids and prednisone increase the concentration (Oppenheimer & Werner 1966, Braverman et al., 1968, Laurell & Rannevik 1979). The changes by age, sex and steroids are generally inversely related to the corresponding changes of the TBG plasma concentration (Braverman et al., 1968, Laurell & Rannevik 1979).

The plasma concentrations of PA, RBP and retinol are highly correlated and are decreased in malnutrition (Ingenbleek et al., 1975a), liver disease (Smith & Goodman 1971) and inflammation (IV, Ramsden et al., 1978, Moody 1982, Kaspar et al., 1975). In protein deficient rats, the rates of synthesis of RBP and PA are similarly decreased (Peterson et al., 1974). The plasma concentrations of PA and RBP are considered more sensitive monitors of changes in the nutritional state than, for example, the plasma concentrations of albumin and transferrin (Ingenbleek et al., 1975b). In liver cirrhosis, the plasma concentration of PA is decreased and reflects the severity of the disease more closely than does the albumin level (Teppo & Maury 1983). The factors responsible for changes in the plasma concentrations of PA, RBP and retinol in inflammation have been the subject of several of the present studies (I, III, IV) and will be discussed later but, briefly, the rate of synthesis of PA seems to be the most important regulator of the plasma concentrations of these components during inflammation.

It may be significant that the "low T_3 syndrome" is found in all the above disorders with low plasma concentration of PA (Chopra et al., 1978); in this syndrome the normal thiol-dependent conversion of T_4 to T_3 is decreased and relatively more rT_3 is formed (Chopra et al., 1978). It is not known, however, whether PA, with its highly reactive thiol groups (V), is involved in the peripheral conversion of T_4 .

In both vitamin A deficiency and renal failure, there is a lack of correlation between the plasma concentrations of PA and RBP. In the absence of vitamin A, RBP is not secreted from rat hepatocytes (or only in very small amount), whereas the secretion of PA is unaffected (Navab et al., 1977b). In renal failure, the glomerular filtration of RBP decreases and thus its concentration increases, whereas the PA metabolism is unaffected (Smith & Goodman 1971, Vahlquist et al., 1973).

High plasma concentration of PA is not generally seen in disease - with the exception of PA secreting tumors (Jacobsson et al., 1979). Unsystematic observations, in our routine laboratory work, of high plasma concentrations of PA in alcoholics could not be confirmed by a systematic study, in which the PA concentration in chronic alcoholics without advanced liver cirrhosis was found slightly, but not significantly elevated (Felding, Fex & Wadstein, unpublished). Similar results were later reported by others (Leo & Lieber 1982, Dickson et al., 1983).

Inherited low plasma concentrations without general disease probably do not occur, although some observations in families with amyloidosis might be interpreted as indicating such conditions (Benson & Dwulet 1983). A genetic relationship between α_1 -antitrypsin deficiency and low plasma concentration of PA (Premachandra & Yu 1979) has been disproven (Felding et al., 1980). Inherited high PA concentrations (about twice the normal value), combined with high concentrations of thyroxine in euthyroid patients have been described (Moses et al., 1982).

There have been many studies in which the plasma concentration of PA has been measured (usually to monitor nutritional state) without, to my mind, justifying the adoption of such analysis in clinic routine, largely owing to the unspecificity of a changed PA concentration.

Prealbumin in cerebrospinal fluid

The CSF:plasma ratio of concentrations is more than 10 times higher for PA than for albumin, RBP and many other plasma proteins (Felgenhauer 1974). This is explained by a dual origin of PA in CSF. An "albumin-like" transport of PA from plasma to the subarachnoidal space delivers the amount expected with regard

to the molecular size and the plasma concentration of PA, but the major part of PA in CSF is secreted to the cisternal fluid, probably mainly by the choroid plexus (Hill et al., 1959, Weisner & Kauerz 1983, Weisner & Roethig 1983). It is not known whether PA is synthesized in the brain or in the choroid plexus. Using immunochemical methods, it can be demonstrated within most cells of the choroid plexus in adults and in advanced fetuses, where other plasma proteins are less abundant (Aleshire et al., 1983, Jacobsen et al., 1982). The presence of PA in the endoplasmatic reticulum and in the Golgi apparatus has been taken as an evidence for its synthesis in the choroid plexus (Aleshire et al., 1983). The molecular properties of PA in CSF have been thought to be different from those in plasma, with regard to electrophoretic mobility (Jeppsson et al., 1979), isoelectric pH (Stibler 1978), and antigenic determinants (Laurell 1972). However, there is no longer reason to believe in molecular forms of PA specific for CSF (V, Felding 1984). The high concentration of PA in CSF indicates that PA may have a specific function in the brain or CSF, the nature of which is not yet known.

The thymic-hormone-like activity of prealbumin

Ninety-five per cent of the ability of human blood to induce T-cell markers in spleen lymphocytes from thymectomized mice has been traced to PA, using general protein separation methods and an azathioprine-sensitive rosette assay (Burton et al., 1978). This activity was also found in mouse PA and decreased without decrease of the PA concentration in thymectomized mice. Further, it was not present in the sera of elderly humans and disappeared from material containing PA after absorption with antibodies to FTS (facteur thymique sérique), a low molecular weight peptide with thymic hormone activity (Bach et al., 1979). This activity may therefore be a function of a low molecular weight ligand of PA as yet unrecognized. To my knowledge, no further progress has been made in this interesting area of PA research during recent years. Finally, it may be mentioned that the impaired cellular immunity in malnutrition is correlated to decreased thymic hormone activity (Chandra 1979) and that the PA plasma concentration is low in this condition.

Prealbumin in amyloidosis

A characteristic feature of all amyloid fibrils is the β -pleated sheet conformation. The amyloidoses are therefore also known as the β -fibrilloses (Glenner 1980). As PA contains a high amount of β -pleated sheet structure, its presence in some types of amyloid fibrils is not perhaps surprising. Since 1978 (Costa et al., 1978), PA related proteins have been recognized as specific amyloid

proteins in all cases investigated of families (differing in ethnic origin) with familial amyloidotic polyneuropathy, FAP type I (Benson 1981, Tawara et al., 1983, Pras et al., 1983, Skinner & Cohen 1981, Libbey et al., 1984), and in hereditary amyloidosis with other symptoms (Benson & Dwulet 1983). These types of amyloidosis are inherited in an autosomal dominant manner. In several of the studies cited here, differences in the amino acid sequences have been demonstrated between normal PA and PA related amyloid protein, one such amino acid substitution - that of normal valine for methionine in position 30 - having also been found in plasma PA from a FAP patient (Dwulet & Benson 1983). A PA related amyloid protein (ASc₁) has even been found in senile systemic amyloidosis, SSA (Westermarck et al., 1977, Sletten et al., 1980, Cornwell III et al., 1981). Preliminary results from studies of Swedish patients with FAP and SSA (Felding et al., 1984) showed a normal distribution, with regard to isoelectric pH, of plasma PA monomers and tetramers. This study also indicated a possible involvement of the cysteinyl group for the binding of PA in SSA and FAP fibrils.

Genetic variation of "prealbumin" between and within species

The structural and functional properties or combination of functions preserved throughout evolution are probably the most important biological features of a protein. The carrier function for vitamin A, via a smaller protein (RBP), has been used to identify analogues to human PA in monkeys (Vahlquist 1972, Vahlquist & Peterson 1972, Van Jaarsveld et al., 1973b), dog (Fex et al., 1977, Fex & Hansson 1980), cow (Fex & Lindgren 1977), goat (Sreekrishna & Cama 1979), rat (Navab et al., 1977a, Peterson et al., 1973), chicken (Bhat et al., 1977, Abe et al., 1975), duck (Sreekrishna & Cama 1978) and the Japanese quail (Heaf et al., 1982). The retinol transport in many different species of vertebrates, including fish, has been studied with regard to the molecular weight of the transporting complex and the affinity of the retinol carrying protein for human PA (Shidoji & Muto 1977). The human pattern was found for all mammals and birds, whereas the fish (carp, shark, young yellowtail) had a low molecular weight retinol-binding protein without affinity for human PA or for proteins in the fish plasma. In the young yellowtail, this protein formed a 1:1 molar complex with retinol, and even in other respects the protein was similar to mammalian RBP. Interestingly, tadpoles showed the piscine pattern of retinol transport, but frogs the mammalian pattern. In the lamprey (a cyclostome), which is considered a very primitive vertebrate, the retinol was transported in ester form with lipoproteins.

When looked for in the studies of mammals and birds mentioned above, a

thyroxine-carrying function and structural similarity to human PA were found for animal "prealbumin" with affinity for RBP. Electrophoretic mobility, however, varied from species to species, as did the immunological properties. At our laboratory special interest has been focussed on the thiol groups of PA, and using the purification procedure involving disulphide-thiol exchange reactions with matrix-bound thiols (Fex et al., 1977), we have demonstrated active thiol groups in PA from man, cow, dog, swine and rat.

Genetic variation of PA within a species was early recognized in monkeys and used to demonstrate the subunit structure by recombination of subunits from PA with different electrophoretic mobility (Robbins et al., 1978). In man, a study of 1900 sera from pregnant women showed only two abnormal sera, both with the same type of abnormal PA. About half of the subunits in this PA had an autosomal inherited abnormal isoelectric pH (Altland et al., 1981). The amino acid substitution already mentioned was subsequently discovered in plasma PA from a patient with familial amyloidotic polyneuropathy (Dwulet & Benson 1983). Both these findings concern structural genes. As mentioned before, conditions with inherited abnormally high PA concentrations have also been demonstrated (Moses et al., 1982).

PRESENT INVESTIGATION

The purpose of the present investigations was generally to expand the knowledge of PA. As it turned out the research concentrated on two subjects:

1. Regulation of the plasma concentrations of PA, RBP and retinol with special reference to PA and the inflammatory condition (papers I-IV) and
2. Reactions of the thiol groups of PA in plasma and CSF (paper V).

Regulation of the plasma concentrations of PA, RBP and retinol with special reference to PA and the inflammatory condition (I-IV)

The plasma concentrations of PA, RBP and retinol are highly correlated over a wide range of concentrations in different conditions (IV, Smith & Goodman 1971, Ingenbleek et al. 1975a).

The aims and means for the regulation of the plasma concentrations of the free or complexed components of the vitamin A transporting system are not known. Study IV indicates that the aim might be a constant plasma concentration of free (non PA-bound) holo RBP.

Because of the binding of RBP to PA in plasma and catabolism of RBP from the free (non PA-bound) fraction of RBP by glomerular filtration (Fig. 2) the effect of changed rate of PA or holo RBP secretion on the plasma concentrations of these components may be difficult to overview. The following model may be helpful in this respect. To simplify, retinol is left out.

1. $\frac{(RBP:PA)}{(PA)(RBP)} = K_a = 10 \text{ l}/\mu\text{mol}$
2. $(PA) + (RBP:PA) = PA_{tot}, PA_{tot,n} = 6 \mu\text{mol/l}$
3. $(RBP) + (RBP:PA) = RBP_{tot}, RBP_{tot,n} = 3 \mu\text{mol/l}$
4. catabolic rate of PA = $k_{PA} \times PA_{tot}$
5. catabolic rate of RBP = $k_{RBP} \times (RBP)$

The association constant (K_a) is about the average of published constants for human and rats. The normal total concentrations $PA_{tot,n}$ and $RBP_{tot,n}$ are average values for rats and humans (III,IV). In equations 4-5 the catabolic rate of PA is assumed to be proportional to the total plasma concentration of PA (I, Socolow et al., 1965) whereas the catabolic rate of RBP is assumed to be proportional to the

plasma concentration of free (non PA-bound) RBP. In the more physiological model Fig. 2 the latter proportionality can be obtained by a constant glomerular filtration rate. In steady state the catabolic rate is equal to the rate of synthesis. This equality and the equations 1 to 5 can be used to calculate the change of total concentrations following changes of the rates of synthesis of the proteins (Table II).

Table II

Effects of changes in the rates of synthesis of RBP and PA on the total plasma concentrations in the simple steady state model.

Rates of synthesis, fraction of normal		Total plasma concentrations, fraction of normal	
PA	RBP	PA	RBP
0.5	0.5	0.5	0.33
0.5	1.0	0.5	0.52

According to this model the total concentrations of PA and RBP can be decreased to a similar degree by a decrease of the rate of PA synthesis alone.

In addition to the rate of synthesis or secretion also the rate of degradation and the flow to and from other compartments of the body as well as plasma volume changes influence the plasma concentration of a protein.

Changes of all these flows may occur during inflammation. The great increase of the plasma concentrations of many "positive acute phase proteins" can only be accomplished by an increased rate of synthesis. Theoretically, the other flow changes mentioned should be able to explain the smaller changes in the plasma concentrations of the "negative acute phase proteins" including PA and RBP. Papers I-IV concern the flows and plasma concentrations of the free or complexed components in the vitamin A transporting system with special reference to PA and the inflammatory condition.

Paper I. In this study the radioactivity in plasma and whole body was followed in rats after simultaneous injection of ^{131}I -labelled PA (intravenously) and croton oil (subcutaneously). The plasma concentrations of PA and orosomucoid as well as the hematocrit and weight were also followed. Normal rats and rats deprived of food from the time of injection of radioactivity were studied simultaneously using the same methods. The results were interpreted according to a non steady state model (Matthews 1965).

The inflammatory reaction induced by croton oil caused changes in the plasma concentrations of PA (decrease) and orosomucoid (increase). These changes were both maximal after 48 h. The intravascular fraction of PA decreased early in the inflammation with a maximal deviation from normal after

about 24 h. The fractional catabolic rate was unchanged. (The normal biological half-life was 12-19 h depending on the method of calculation.) The results indicated a decreased rate of PA synthesis, but in the non steady state model it was difficult to quantitate the decrease. In conclusion, the inflammatory decrease of the plasma concentration of PA seemed to be due to an early redistribution of PA to the extravascular compartment combined with a decreased rate of PA synthesis.

In the fasting rats a decreased rate of synthesis seemed to be responsible for the measured decreased plasma concentration of PA. A great deviation from normal with regard to plasma volume and elimination of non protein-bound radioactivity made the comparison to normal less reliable for these rats, although corrections for such deviations were included in the calculation.

After the paper (I) had been submitted, an Australian group (Dickson et al., 1982) reported similar tracer experiments. By following the amount of PA in whole rat homogenates as an additional parameter they probably more reliably demonstrated a decreased rate of PA synthesis during acute inflammation.

In the meantime we had turned to the system of isolated rat hepatocytes in primary monolayer cultures to more directly study changes in the rates of synthesis of PA and RBP under different conditions (II,III). In both studies the results were based mainly on immunochemical determinations of the concentrations of the proteins in incubated medium and cell lysates.

Paper II. The purposes of this study were to learn the technique and to determine the hepatic fraction of the whole body production of PA.

Our isolated hepatocytes synthesized PA and albumin in the same ratio as that previously found for the whole body synthesis in vivo by steady state turnover experiments with radiolabelled proteins in the rat (I, Peterson et al., 1974, Matthews 1957). This could be shown also without using absolute protein concentrations, thus avoiding the possible inaccuracy from such determinations. Albumin is exclusively synthesized in the liver (for discussion, see II). Our results therefore would imply a mainly hepatic origin of PA, if the ratio of rates of synthesis of the two proteins in the isolated hepatocytes was similar to that in hepatocytes in vivo. This similarity was made probable by a similar PA:albumin ratio of synthesis in different media and during several days of culture. A similar ratio was also found in the normal isolated hepatocytes in the next study (III) under other culture conditions and with about 3 times higher total rates of synthesis. We therefore believe that this ratio is rather resistant to changes and that it is preserved during the isolation and culture. In conclusion, study II made a mainly hepatic origin of plasma PA in the rat likely.

Paper III. In this study the rates of synthesis of PA, RBP and other plasma proteins were measured in isolated hepatocytes using immunochemical methods as in the previous paper. The experiments were designed to compare these rates in normal hepatocytes with the corresponding rates in hepatocytes from rats with an inflammatory reaction. The inflammatory reaction was induced by croton oil 18 h before the cell isolation procedure. The increase in the medium of the extracellular amount of each protein per day and mass unit of total cell protein was measured for three successive 24 h incubation periods and taken as an expression of the rate of synthesis.

The inflammatory pattern of protein synthesis seemed to persist for one or two days after isolation of the cells. Expected rate changes were observed for albumin, transferrin, α_1 -macroglobulin and orosomucoid. The inflammation decreased the synthesis of both PA and RBP but significantly more so for PA, although the RBP serum concentration was more decreased than the serum concentration of PA at the time of the cell isolation. During the first incubation the normal cells synthesized RBP and PA in a molar ratio RBP:PA of about 1:1. This is similar to the normal in vivo ratio of synthesis (Peterson et al., 1974) (demonstrating the hepatic origin of the major part of RBP).

In the former paper (II) the preservation of the in vivo relation between the rates of PA and albumin synthesis was of fundamental importance for the conclusion. In this paper (III) such preservation was of less importance if only the changes in the pattern of protein synthesis induced by the isolation and culture were similar for the "inflamed" and normal hepatocytes. As an example, addition of dexamethasone and insulin to the medium increased the rates of albumin and orosomucoid, and slightly the rate of PA synthesis, similarly in "inflamed" and normal cells. The inflammatory changes could therefore still be detected in the hormone-treated cells.

In conclusion, the previously suggested decreased rate of PA synthesis during acute inflammation was further made probable. The rate of RBP synthesis was less affected in spite of the low plasma concentration of RBP. Thus the results support the idea that the plasma concentration of PA, RBP and retinol during inflammation might be mainly determined by the rate of PA synthesis (as illustrated in Table II).

Paper IV. In this study we measured the total concentrations of PA, RBP and retinol in plasma from normal men and from patients with various chronic inflammatory conditions. Patients with liver cirrhosis and renal failure were excluded.

The concentration of free (non PA-bound) holo RBP was then calculated from the equations given in paper IV.

The total plasma concentrations of the components measured were all decreased in the patients, whereas the calculated free holo RBP concentration was normal. Further, the total concentration ratio RBP:PA (and the free RBP concentration) was increased in the patients.

Because of their chronic condition the patients can be considered in steady state. According to the principles in the model given in connection with Table II the results indicated an unchanged or even increased rate of RBP synthesis in combination with a decreased rate of PA synthesis. The aim seemed to be a constant plasma concentration of free holo RBP.

The free holo RBP plasma concentration was also calculated in patients with liver cirrhosis from data in the literature. It discriminated better than the total plasma concentrations of RBP, PA and retinol between patients with normal and abnormal dark adaptation ability. The free holo RBP therefore seems more directly involved in the vitamin A supply to the tissues than the other components of the vitamin A transporting system.

The role of PA in the vitamin A transport. According to the present knowledge a more detailed model of the vitamin A transport from the liver to the cells ought to be in accordance with the following statements:

1. RBP and retinol are secreted to the circulation mainly as holo RBP.
2. PA is secreted and catabolized independently of RBP.
3. RBP is removed from the circulation mainly by glomerular filtration of the free (non PA-bound) fraction.
4. holo RBP has a higher affinity than apo RBP for PA and for the RBP receptor at the cell membrane.
5. holo RBP (free or PA-bound) is the form in which vitamin A is accepted by the cells.

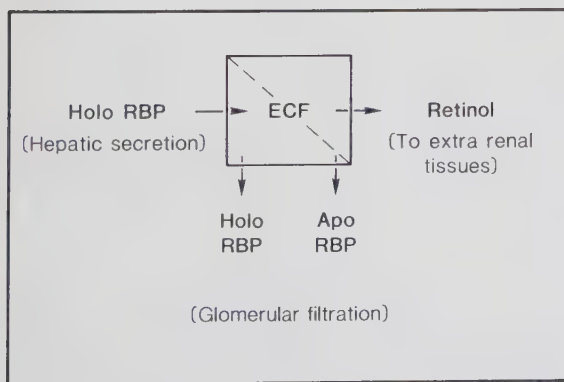


Fig. 3. Retinol turnover. ECF is extracellular fluid. The dashed line indicates the separation between plasma (left) and interstitial fluid (right).

Fig. 3 illustrates the retinol transport. In Fig. 2 PA apparently directs the flow of retinol away from the kidneys and to the extrarenal tissue. However, in a steady state model with one compartment as in Fig. 3 (without dashed line) and with equilibrium in the binding reactions between PA and the two forms of RBP this change is only possible if PA carries retinol all the way to the cell membrane.

Possibly a two-compartment-model (Fig. 3 with dashed line) can allow both a retinol saving effect of PA and a delivery of retinol to the cell from free holo RBP. Thus, PA protects the bound holo RBP in plasma from glomerular filtration. When the complex enters the extravascular compartment (where this protection is not needed) holo RBP is partly liberated owing to the lower total concentrations of the proteins in the interstitial fluid. PA can return to plasma with the lymph, and free holo RBP can proceed to the target cells.

Membrane receptors for free holo RBP and cellular uptake of (^3H) retinol from free holo RBP have been demonstrated in the absence of PA, whereas receptors for PA have not been found (Heller 1975, Bhat & Cama 1979b, Rask & Peterson 1976). Addition of PA to the cell suspension decreased the uptake of (^3H) retinol from holo RBP under experimental conditions, but the decrease seemed less than corresponding to the decrease of the free holo RBP concentration (Rask & Peterson 1976). Thus, possibly the cells can accept retinol both from free and PA-bound holo RBP. This is in somewhat disaccordance with the previously indicated (IV, Bhat & Cama 1979a, Heaf et al., 1982) importance of the free holo RBP for the vitamin A supply to the cells. The relative contribution from each of these forms of holo RBP to the saturation of the holo RBP receptors in vivo is not known. The degree of this saturation is also unknown.

At the present state of knowledge the vital importance of PA for the vitamin A transport, if any, is difficult to assess. It is likely that the PA-holo RBP complex mainly serves as a source of readily available holo RBP, in which retinol is protected from glomerular filtration and possibly has a further protection against oxidation in addition to that given by RBP alone. I find a retinol saving effect of PA and a direct delivery of holo RBP to the cell receptor from the PA-holo RBP complex possible, but less supported.

Plasma concentrations are generally the most easily obtained "turnover" data. It is therefore of interest to extract maximal information from such concentrations. The following can be considered as a play with a model, but some principles of the vitamin A turnover are illustrated.

Steady state and the above statements (especially 1 and 3) lead to the conclusion that the rate of extrarenal (i.e. extratubular) uptake of vitamin A in the body is equal to the glomerular filtration rate of apo RBP, i.e. proportional to the plasma concentration of free apo RBP. The steady state

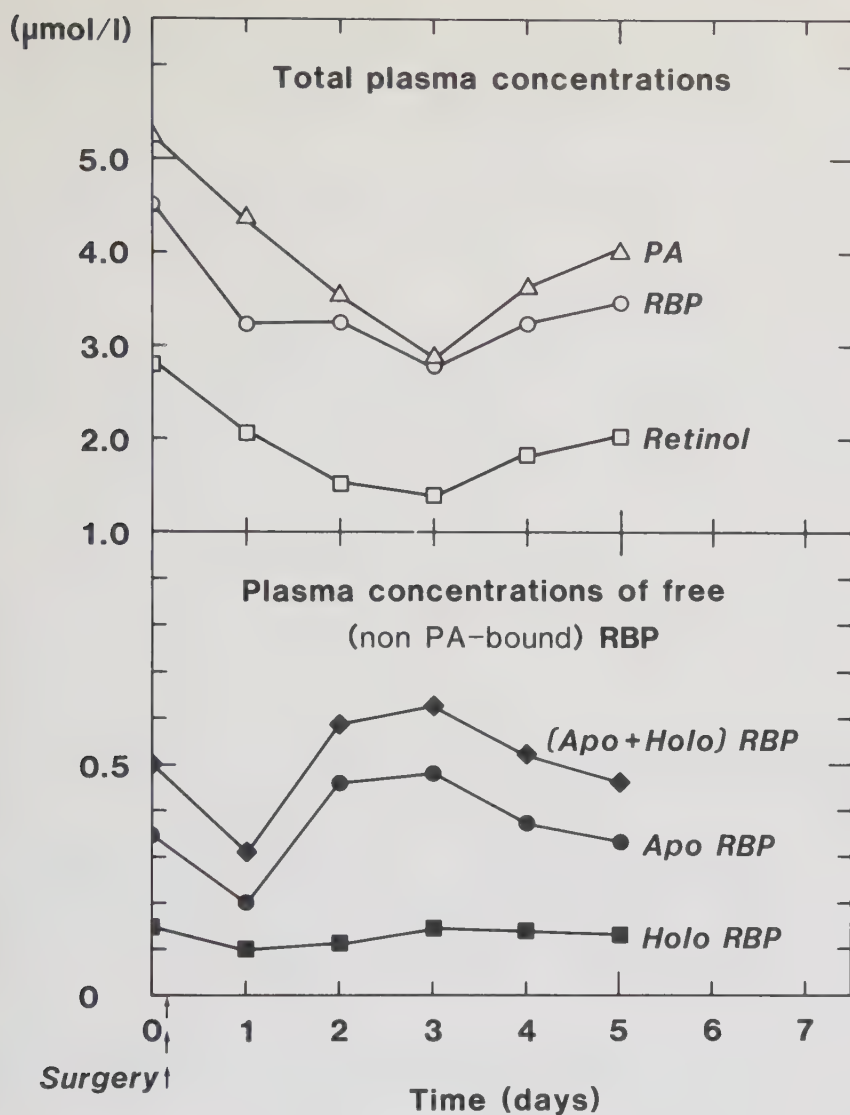


Fig. 4. Post-surgical changes in total plasma concentrations of PA, RBP and retinol taken from Ramsden et al., 1978 (mean of 11 patients). The plasma concentrations of free apo and holo RBP were calculated from these mean values by the equations in IV.

Table III

Plasma mean concentrations, $\mu\text{mol/l}$, in 20 normal men and 60 patients with chronic inflammation (IV).

Diagnosis	Total concentrations			Conc. of free* RBP forms		
	PA	RBP	Retinol	apo RBP	holo RBP	RBP
Normal	5.35	3.86	2.92	0.149	0.119	0.268
Inflammation	3.94	3.08	1.81	0.268	0.104	0.372

* Free (non PA-bound) apo and holo RBP concentrations, calculated from the mean total concentrations by the equations in IV.

Table IV

Plasma mean concentrations, $\mu\text{mol/l}$ during nutritional treatment of 37 children, aged 18-30 months, with severe protein-calorie malnutrition.

Day	Total concentrations*			Conc. of free** RBP forms		
	PA	RBP	Retinol	apo RBP	holo RBP	RBP
1	1.16	0.77	0.40	0.136	0.046	0.182
8	2.38	1.68	0.90	0.199	0.065	0.264
15	3.54	2.35	1.31	0.193	0.065	0.258
22	4.06	2.58	1.47	0.178	0.061	0.239

* Taken from Ingenbleek et al. 1975b.

** Free (non PA-bound) apo and holo RBP concentrations, calculated from the mean total concentrations by the equations in IV.

model given in connection with Table II can then be extended to:

rate of PA synthesis = K_{PA} x total plasma concentration of PA,

rate of RBP synthesis = K_{RBP} x plasma concentration of free RBP

rate of extratubular retinol uptake = K_{RBP} x plasma concentration of free apo RBP

In addition, as discussed above, the plasma concentration of free or bound holo RBP may reflect the availability of a vitamin A for the cells.

The concentrations mentioned can be calculated from the total plasma concentrations of PA, RBP and retinol using the equations given in IV. K_{RBP} can be considered equal or proportional to the glomerular filtration rate (GFR). In the interpretation of the above Tables (III and IV) and Fig. 4 it should be remembered that steady state and equal glomerular filtration rates in compared situations are not always present. The most reliable comparison is probably found in Table IV, day 8 to 22. The main conclusions drawn from Tables III and IV and Fig. 4 are:

The rate of synthesis of PA in disease is decreased more than the rate of synthesis of RBP; the rate of RBP synthesis is only decreased in extreme situations (untreated severe protein-calorie malnutrition and probably the first day after surgery); other factors than the plasma concentrations of free or PA-bound holo RBP seem important for the rate of extra renal retinol uptake; a retinol saving effect of PA illustrated by an increase of the ratio free apo RBP:free holo RBP with increasing plasma concentration of PA was not generally found.

Reactions of the thiol groups of prealbumin in plasma and cerebrospinal fluid (V)

Protein thiol groups, reactive in thiol-disulphide interchange reactions, are rare in plasma. The major part is found in albumin, α_1 -antitrypsin and PA. According to the present knowledge (V), PA contains the highest number of these groups per mass unit. In the considerable literature on PA, part of which is reviewed in the "Introduction", this feature of PA plays a very little part. The four cysteinyl residues in purified PA were early considered to be unreactive with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) or iodoacetic acid due to steric hindrance (Rask et al., 1971) or intramolecular disulphide bonds (Raz et al., 1970). After mild reduction two of the four cysteinyl groups reacted with DTNB (Raz et al., 1970). All four cysteinyl residues seemed to be accessible to alkylation or reactive with DTNB in 6.5 mol/l isopropanol under denaturing conditions with or without reduction (Rask et al., 1971).

The unreactivity under non denaturing conditions is surprising since complexes between PA and light Ig chains of κ -type can be detected in plasma from myeloma patients with Bence Jones proteinuria (Laurell & Thulin 1975). PA in plasma also reacted with DTNB and with the mixed disulphides formed by the reaction of DTNB with free or matrix-bound thiols (Laurell & Thulin 1975, Fex et al., 1977). This demonstrated reactive thiols in native plasma PA, but the number of such groups per molecule PA was not determined.

We (Felding & Fex) have generally confirmed the above referred results concerning the reactivity of the thiol groups in purified, stored PA with one, probably important exception. With our denaturing system (0.01 mol/l phosphate buffer containing 6 mol/l guanidine hydrochloride and 0.005 mol/l EDTA at pH 5.9) a reducing agent was a prerequisite for the subsequent alkylation of all cysteinyl residues. We used 0.1 mol/l dithiothreitol (DTT) for the reduction. After dialysis the alkylation by (^{14}C)-iodoacetic acid was performed at pH 8.5 (still in 6 mol/l guanidine hydrochloride). PA purified from plasma and PA purified from CSF behaved similarly in these analyses. We were not able to form a hypothesis, which consistently explained our results. This was particularly difficult if also the results mentioned in the literature should be accounted for.

Simultaneously, I studied the microheterogeneous distribution of PA with regard to isoelectric pH (PI) using the electrofocusing techniques described in paper V. Tetrameric (non-denatured) PA showed a complex pattern difficult to interpret. The purification of PA from both CSF and plasma increased the number of bands and made the distribution more acidic. As noticed previously (Stibler 1978) we also found PA in CSF more acidic than PA in plasma. By the reaction of a protein-thiol with DTNB the protein receives an additional negative charge (at the pH values relevant here). Charge changes by reaction with DTNB could therefore be used to evaluate the thiol reactivity. We generally found that the thiol reactivity decreased with decreasing PI. Still, however, the results were difficult to interpret. A break-through in this research was the use of electrofocusing in 8 mol/l urea. By this technique only two PA-bands were formed when plasma and CSF were applied to the gel (V, Fig. 1). The major band had pI 5.7 and the minor band pI 5.4. The same main bands (and additional more acidic bands) were found in purified PA from plasma and CSF. When I first developed this technique we were unable to convincingly prove that these bands represented monomers of PA with different charge. With a similar technique and by use of a genetic variant of PA, the bands were later shown to be monomers (Altland et al., 1981). I also arrived at this conclusion mainly from the identical relative distribution of the radioactivity after electrofocusing in

8 mol/l urea of partly and totally (^{14}C)-carboxymethylated PA. The two forms of PA monomers are also seen in two-dimensional electrophoresis of plasma (Anderson et al., 1982). By electrofocusing in urea it became much more easy to interpret the pI changes seen after reactions of the thiol groups. The acidic (anodal) subunit (pI 5.4) did not react with DTNB, whereas the cathodal subunit (pI 5.7) reacted almost quantitatively in both plasma and CSF. This indicated that the thiol group of the acidic subunits had already been modified and that this modification caused their higher negative charge and their unreactivity towards DTNB. The acidic subunits constituted less than 10 per cent of PA in fresh samples of both plasma and CSF. Their relative amount increased more rapidly in CSF than in plasma by storage of the samples. This explains the higher negative charge (Stibler 1978) and the higher electrophoretic mobility (Jeppsson et al., 1979) of PA in CSF.

Paper V describes the above reactions of the thiol groups of PA during storage of plasma and CSF. It was made probable that the reactions included oxidation without formation of disulphide bonds. The chemistry of the reactions was not revealed in detail. A new approach to the determination of protein thiol pK values was used (V, Fig. 3) and can probably be further developed.

Paper V concerns only PA in plasma and CSF, not the purified PA from these sources. The reactions considered in paper V may explain the decreased reactivity of the thiol groups also in purified PA. However, with purified PA the relation between PI and thiol reactivity seemed more complex.

SUMMARY

The major parts of PA and RBP in rat plasma were found to be of hepatic origin. The secretion of holo RBP and PA was not linked. During acute inflammation in the rat the plasma concentration of PA decreased due to decreased synthesis combined with redistribution of PA to the extravascular compartment. The plasma concentration of RBP in rat and human during inflammation seemed mainly regulated by the plasma concentration of PA. The concentration of free (non PA-bound) holo RBP was found to be normal in chronic inflammation in man in spite of the decreased total concentrations of RBP, PA and retinol. The free holo RBP concentration also better than these total concentrations discriminated between patients with normal and abnormal dark adaptation (calculated from data in the literature). These observations indicate that free holo RBP might be both the regulated parameter and the direct supplier of vitamin A to the tissues.

PA contains four cysteinyl residues. More than 90 per cent of the cysteinyl residues of PA in fresh plasma and CSF were found highly reactive in thiol

disulphide exchange reactions, for example with DTNB. Both in vitro and in vivo the cysteinyl residues seemed to receive an additional negative charge and become unreactive with DTNB. In vitro these reactions proceeded faster in CSF than in plasma explaining the previously recognized higher negative charge and higher electrophoretic mobility of PA in CSF. The chemistry of these reactions was not revealed in detail, but it seemed as if oxidation without formation of disulphides was involved. These reactions might explain the known unreactivity of the cysteinyl residues in purified PA.

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Factors responsible for the decreased plasma concentration of prealbumin during acute inflammation and fasting in the rat

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The concentration of prealbumin in plasma and the elimination from plasma and whole body of intravenously injected ^{131}I -labelled rat prealbumin were followed in normal rats and rats subjected to fasting or acute inflammation. During fasting and inflammation the concentration of prealbumin decreased to about 50% of the initial value. It was concluded from the radioactive tracer experiments that decreased synthesis was responsible for the decreased intravascular prealbumin mass in the fasting rats. In these rats the plasma volume also decreased. In the rats subjected to inflammation, both a decreased intravascular fraction and a decreased synthetic rate of prealbumin seemed to contribute to the decreased intravascular mass of prealbumin. The total catabolic rate was decreased in these conditions.

Key words: Prealbumin, radio-iodinated, metabolism, inflammation, fasting

Human prealbumin is one of the thyroxine-transporting plasma proteins and it is also, indirectly involved in the transport of vitamin A in plasma (Goodman 1976, Robbins et al. 1978). The plasma prealbumin concentration is low in malnutrition (Ingenbleek et al. 1975, Gofferje 1978, Young & Hill 1978, Young et al. 1979), inflammation (Socolow et al. 1965, Harris & Kohn 1974, Aronsen et al. 1972) and liver disease (Smith & Goodman 1971, Hutchinson et al. 1981, Kindmark & Laurell 1972). Rat prealbumin has similar properties and functions (Navab et al. 1977, Scherer et al. 1977, Peterson et al. 1974). A decrease of the amount of prealbumin in plasma can theoretically be caused by decreased synthesis, increased elimination or altered distribution of prealbumin. The purpose of this paper was to determine the relative importance of these mechanisms during decreasing plasma prealbumin concentration in fasting and acute inflammation.

MATERIALS and METHODS

Labelled rat prealbumin. Rat prealbumin purified as previously described (Fex et al. 1977) was labelled with ^{131}I

by the lactoperoxidase method (Thorell & Johansson 1971) using Na^{131}I , 10–19 mCi/ μgI (The Radiochemical Centre, Amersham, England). The labelled protein was separated from excess iodine by passage over a Sephadex G-25 column (1.5 $\times$ 5.7 cm). The number of iodine atoms per prealbumin molecule was less than 0.5 at this stage. The labelled prealbumin was then subjected to affinity chromatography on a column (1.5 $\times$ 5.0 cm) with human retinol-binding protein coupled to Sepharose (Fex et al. 1977). The ^{131}I -labelled rat prealbumin with affinity for human retinol-binding protein was used within 4 hours of labelling. The fraction of the radioactivity precipitated by 12.5% (w/v) trichloroacetic acid containing 0.5–1.0 mM NaI ranged from 0.80 to 0.94. The labelled product diluted with rat plasma had the same mobility as the native prealbumin on agarose gel electrophoresis at pH 8.6.

Plasma protein concentrations and haematocrit. The plasma concentrations of prealbumin and orosomucoid were measured by electroimmunoassay (Laurell 1972) using rabbit antisera and with pooled rat serum as calibration standard. The haematocrit was measured in heparinized precision capillary tubes after centrifugation for 10 min at 10 000 $\times$ g. The intravascular mass of a protein was considered identical to the concentration of the protein in plasma $\times$ plasma volume.

Experimental procedure. Male Sprague-Dawley rats weighing between 174 and 248 g were used. During 3–7 days preceding the experiment they received 0.06–0.1 mmol KI per day with the food and had free access to

water with 0.6–1.2 mmol KI per litre. ^{131}I -labelled prealbumin (20–97 μCi , 3–15 μg) in 0.4–0.6 ml saline was injected into the left jugular vein under ether anesthesia. In each experiment a fasting rat and/or a rat with induced inflammation was investigated together with one normal rat. The rats used in the same experiment had initially similar body weight and concentration of plasma proteins. Each rat was placed alone in a "metabolic cage" allowing collection of urine and faeces. During the experiment all rats had access to the above-mentioned drinking water with KI and also to 10% (w/v) NaCl from a separate bottle. The normal rats and the rats with induced inflammation were given ordinary food pellets without addition of KI. Inflammation was induced by injection of croton oil subcutaneously in the gluteal region on both sides (totally 0.1 ml) immediately after the administration of tracer. The rats to be fasted were deprived of food the first 90–95 h after injection of the tracer. Thereafter they received ordinary food pellets (without KI). Blood was collected in heparinized capillary tubes from an incision in the tail. About 300 μl blood corresponding to about 2% of the blood volume was taken eight times during the first four days of the experiment and 1–3 times during the next three days. After measurement of hematocrit the capillary tubes were cut and the plasma collected and kept at -20°C until the end of the experiment. The whole body radioactivity was measured after urination just before each blood sampling.

Determination of radioactivity. For determination of whole body radioactivity the rat was placed in a narrow plexiglass cage at a constant position in relation to the NaI crystal. A 200 ml cylindrical plastic flask which fitted precisely into the plexiglass cage and contained 200 ml buffer with albumin and the same volume of the tracer solution as the rats was counted together with the rats. At the end of each experiment aliquots from this flask solution and the plasma samples were mixed with trichloroacetic acid. After centrifugation (2000 $\times$ g, 15 min) the precipitates and supernatants were counted in a LKB Wallac 1280 Ultragamma. The counting error for the radioactivity in whole body and precipitates from plasma or flask solution were always less than 2%.

Calculation of tracer retention curves and plasma volume. The protein-bound radioactivity in both plasma and the whole body was expressed as fraction of the protein-bound radioactivity in the injected dose using the mentioned 200 ml flask as the link between the two counting systems. The whole body radioactivity multiplied by the ratio of trichloroacetic acid precipitable radioactivity to total radioactivity in plasma to the same time was taken as an estimate of the protein-bound radioactivity in the whole body. During inflammation the ratio of protein-bound radioactivity to total radioactivity in plasma was rather constant and did not change from normal. However in the fasted rats ($n=5$) the mean value $\pm$ 1 SD of this parameter decreased from 0.94 ± 0.02 to 0.75 ± 0.05 during the first 65 h. As initial plasma volume we used 40 ml/kg, which was the extrapolated value at zero time of the distribution volume of prealbumin-bound ^{131}I measured once or twice in the 18 rats in the period 5–50 min after i.v. injection. Assuming a constant intravascular red cell volume the plasma volume at time t (PV_t) was derived¹

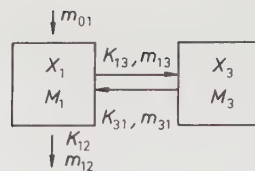


Fig. 1.

X_j = Protein-bound radioactivity as fraction of injected protein-bound radioactivity, in pool j to time t .

M_j = Mass of prealbumin in pool j .

m_{ij} = Flow of prealbumin (mass/time) from pool i to pool j .

$k_{ij} = \frac{m_{ij}}{M_i}$ = Fractional rates.

$S_j = \frac{X_j}{M_j}$ = Specific activity in pool j .

1 = Intravascular pool.

2 = Eliminated protein.

3 = Extravascular pool.

from the initial plasma volume (PV_i) and the hematocrits (Hct_i and Hct_t).

Statistical analysis. For statistical analysis the values of selected parameters from each fasting rat and each rat with inflammation were expressed as fractions of the corresponding values for the normal rat from the same experiment and time. For each experiment and condition (fasting or inflammation) the mean of these ratios (fractions of normal values) was calculated for the period of decreasing prealbumin concentration in plasma. From the mean ratios obtained from different experiments the overall mean ratio was calculated and compared to 1 in a t -test (degree of freedom = number of experiments – 1).

Interpretation of tracer experiment. The interpretation followed the outlines of Matthews (1965), who used the model in Fig. 1 for analysis of non-steady state experiments.

RESULTS

The mass of prealbumin and orosomucoid in plasma, the body weight and the haematocrit are given in Fig. 2 as fractions of the initial value. The mass of prealbumin reached a minimum and the mass of orosomucoid as maximum 40–43 h after induction of inflammation with croton oil. During fasting the body weight and the mass of prealbumin in plasma declined till food was given after 90–95 h. Including initial studies, in which only protein concentrations were measured, the mean concentration $\pm$ 1 S.D. of

¹ $\text{PV}_t = \text{PV}_i \cdot \frac{\text{Hct}_i / 1 - \text{Hct}_t}{\text{Hct}_t / 1 - \text{Hct}_i}$

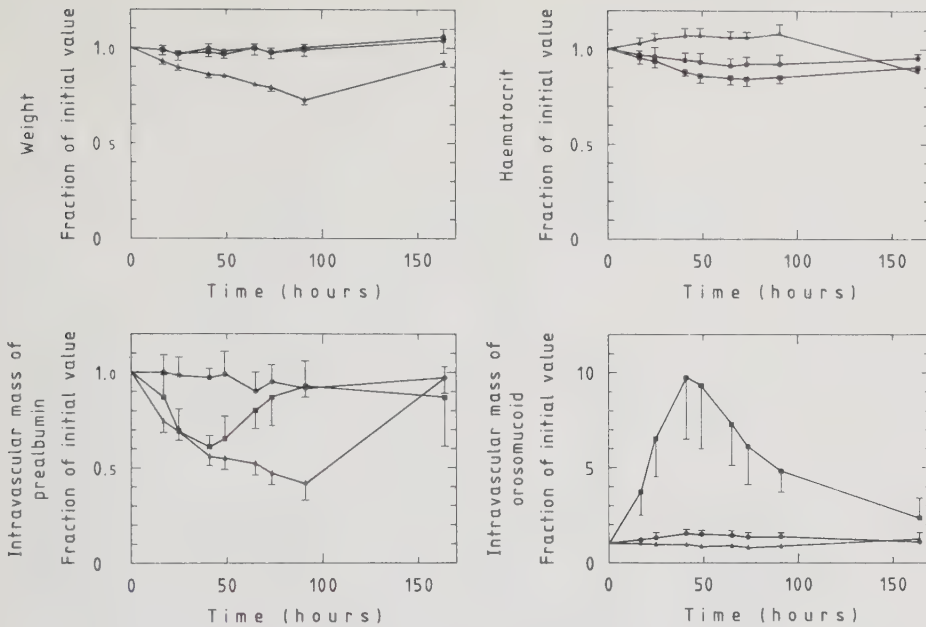


Fig. 2. Mean $\pm$ 1 S.D. of some variables during the experiments. ●, normal rats; ▲, fasting rats; ■, rats subjected to inflammation. Inflammation and fasting were started at time zero. Food was given to the fasting rats after 91 h. Each mean value in the period 0–93 h represents 4–7 rats. The standard deviation is only shown, when it exceeds the range of the symbols

prealbumin in plasma was $50 \pm 5\%$ ($n=8$) of the initial value after 48–50 h of inflammation and $52 \pm 6\%$ ($n=7$) after 72–75 h fasting. The time course of several tracer-related parameters during fasting and inflammation are shown together with the normal curves in Fig. 3. Except for the increased retention of tracer in the extravascular space during fasting the changes from normal of the tracer retention curves seem rather small during fasting and inflammation. The ratios fasting/normal and inflammation/normal calculated for tracer related parameters and intravascular mass of prealbumin are shown in Table 1. The measured parameters are listed in the left column. The ratio fasting/normal and inflammation/normal of these parameters calculated as described in the method section are given in the next columns. The right hand side of the table is purely theoretical. In the model, (Fig. 1) we have selected those rate changes which will result in a decrease of the intravascular mass of prealbumin. These rate changes are decreased syn-

thesis ($m_{01} \downarrow$), changed distribution rates ($K_{13} \uparrow$ and/or $K_{31} \downarrow$) and increased catabolism ($K_{12} \uparrow$). The theoretical qualitative effect in the considered period of each of these rate changes on each of the measured parameters is given by + (increase), 0 (no change) or – (decrease). Signs in parenthesis, (+) and (–), indicate that the changes are secondary and small. As an example, an increase of the fractional catabolic rate ($K_{12} \uparrow$) will theoretically decrease the intravascular mass of prealbumin, decrease the whole body protein-bound radioactivity, decrease the intravascular protein-bound radioactivity, decrease the intravascular fraction of the whole body protein-bound radioactivity to a small extent, increase the measured fractional catabolic rate and increase the total catabolic rate. As seen to the left hand side of the table such a pattern of changes from normal are found neither during fasting nor during inflammation indicating that increased fractional catabolic rate cannot alone explain the results. The change from normal of the

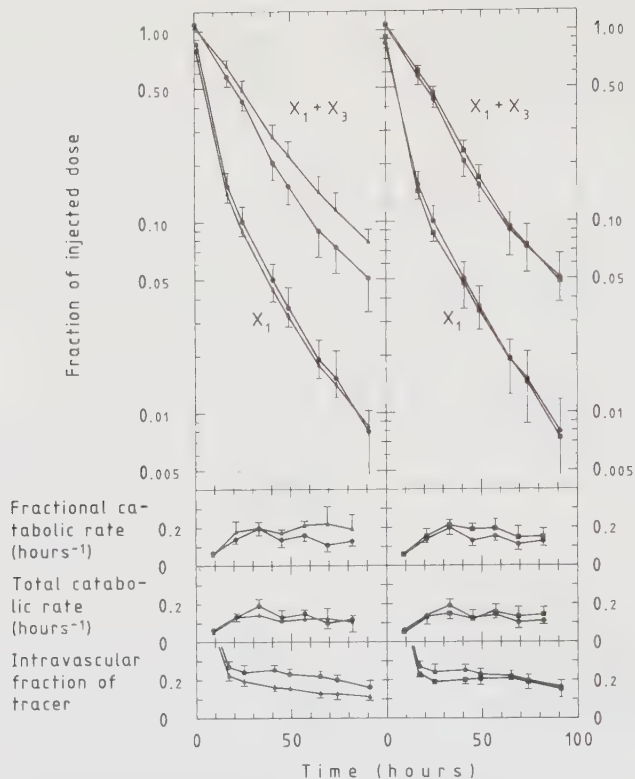


Fig. 3. Results obtained after intravenous injection of ^{131}I -labelled rat prealbumin into 6 normal rats, $\bullet$ (left and right); 5 fasting rats, $\blacktriangle$ (left) and 4 rats subjected to inflammation, $\blacksquare$ (right). Fasting and inflammation were started at time zero. X_1 and X_3 are intra- and extravascular protein-bound radioactivities respectively. Fractional catabolic rate was calculated as the decrease in whole body radioactivity ($X_1 + X_3$) divided by the period of time (Δt) and the mean intravascular activity ($\bar{X}_1$) in the period. Total catabolic rate was calculated as fractional catabolic rate multiplied by the mean intravascular mass of prealbumin expressed as fraction of the initial value (see Fig. 2). Intravascular fraction of tracer is $X_1/(X_1 + X_3)$.

measured parameters during fasting cannot be explained by any of the considered theoretical rate changes alone or in combination. However, the changes observed during inflammation at the 5% significance level ($\alpha=0.05$) can be explained qualitatively by changed distribution rates alone or in combination with decreased synthesis. At the 2.5% significance level decreased synthesis alone can explain the decreased intravascular mass of prealbumin during inflammation.

DISCUSSION

The real whole body (and extravascular) tracer retention curves during fasting may be more close to the normal curves than those calculated by our method. Because of different molecular size of the I^- -ion and the plasma proteins the ratio of free ^{131}I to protein-bound ^{131}I is probably higher in the extravascular compartment than in plasma. This will result in an overestimation of the whole body protein-bound radioactivity by our method of calculation.

Table 1. Experimental results and effects of rate changes in the theoretical model (Fig. 1) in the period of decreasing intravascular mass of prealbumin

-, 0, +, symbolize decrease, no change and increase respectively. (-) and (+) indicate that the changes are secondary and small

Measured parameter	Ratios of measured parameter values from paired rats ^a				Theoretical change from normal of the parameter in the left column following selected rate changes in the model		
	Fasting/normal		Inflammation/normal		Decreased synthesis ($m_{01} \downarrow$)	Increased catabolism ($k_{12} \uparrow$)	Changed distribution rates ($K_{13} \uparrow$ and/or $K_{31} \downarrow$)
	Mean	1 SD (n=5)	Mean	1 SD (n=4)			
Intravascular mass M_1 of prealbumin	0.69 ^b	0.10	0.74 ^b	0.11	-	-	-
Whole body protein-bound radioactivity ($X_1 + X_2$)	1.44 ^b	0.31	1.03	0.05	0	-	(+)
Intravascular protein-bound radioactivity (X_1)	0.99	0.10	0.86 ^b	0.10	0	-	-
Intravascular fraction of protein-bound radioactivity ($X_1/(X_1 + X_2)$)	0.72 ^b	0.09	0.83 ^b	0.13	0	(-)	-
Fractional catabolic rate	1.21 ^b	0.14	1.17	0.22	0	+	0
Total catabolic ^c	0.82 ^b	0.09	0.85 ^b	0.10	-	+	(-)

^a The values represent the interval from 16 to 65 h after start of the experiments for the fasting rats and from 16 to 41 h for the rats subjected to inflammation. See methods for details

^b The change from 1 is significant in a one tailed t-test at the 5% significance level.

^c Calculated as in Fig. 3 ($\frac{\Delta(X_1 + X_2)}{X_1 + X_2}$ and $\frac{\Delta(X_1 + X_2)M_1}{X_1 + X_2}$ respectively).

tion. The overestimation will increase with decreasing protein-bound fraction of the radioactivity in plasma and will therefore be greater in the fasting rats than in the other rats. A double tracer technique (Bianchi et al. 1973) could only to some extent have solved these problems. For the fasting rats the results in Table 1 could not be explained consistently by any rate changes in the model (Fig. 1). If we, for above-mentioned reasons, do not rely on the whole body tracer retention curve, then only the intravascular protein-bound radioactivity and intravascular mass of prealbumin are reliable parameters for the fasting rats in Table 1 and only decreased synthesis ($m_{01} \downarrow$) can explain the results.

The physiological interpretation of the suggested distribution rate changes during inflammation would most probably be an increased capillary permeability ($K_{13} \uparrow$). In an additional experiment we injected 500 μCi ^{131}I -labelled prealbumin in v. jugularis and 0.1 ml croton oil subcutaneously in the left thigh. After 20 h a picture (not shown) of the rat

taken with a pin hole gamma camera showed clear accumulation of radioactivity at the site of the croton oil injection. This indicates increased capillary permeability in the inflamed area. To judge whether the changes in Table 1 during inflammation can be explained quantitatively without decreased synthesis we can consider the expression for intravascular specific activity $S_1 = X_1/M_1$. Neither increased capillary permeability ($K_{13} \uparrow$) nor increased fractional catabolism ($K_{12} \uparrow$) has any immediate effect on $\frac{dS_1}{dt}$ in the model (Matthews 1965), whereas de-

creased synthesis causes an increase compared to normal. In computer simulated tracer experiments with albumin in the rabbit (Matthews 1965) the effect on S_1 of 50% increase in K_{13} after distribution of the tracer is hardly seen over a period of about $3 \times T_{\frac{1}{2}}$. This means that if the synthesis is unchanged then we should expect X_1/M_1 to be almost normal in the period of decreasing intravascular mass M_1 of prealbumin. However, as seen from Table 1, M_1 has decreased more than the

intravascular radioactivity X_1 indicating a contribution from decreased synthesis to the decrease in intravascular mass of prealbumin.

The results concerning the protein concentrations during inflammation are in full accordance with those from other investigations on rats (Scherer et al. 1977) and man (Aronsen et al. 1972). The decreased plasma concentration of prealbumin in the fasting rats corresponds to those found in fasting humans (Gofferje 1978) and protein deficient rats (Peterson 1974). The increased haematocrit in fasting rats has also been shown previously (Kitazawa & Komuro 1977). The fractional metabolic rate with the plasma pool as reference has earlier been determined to 0.13 h^{-1} for normal rats and 0.092 h^{-1} for protein deficient rats in steady state (Peterson et al. 1974). These values are of the same magnitude as those in Fig. 3. When the fractional catabolic rate in our normal rats was calculated as plasma tracer retention curve area⁻¹ (Nosslin 1964) using the peel-off technique (Matthews 1957) for area calculation, we found the mean value $\pm 1 \text{ SD} = 0.103 \pm 0.015 \text{ h}^{-1}$ ($n=6$). The intravascular fraction of prealbumin calculated as plasma tracer retention curve area/whole body tracer retention curve area (Nosslin 1964) was 0.35 ± 0.03 (mean $\pm 1 \text{ SD}$) in our normal rats. Using only the plasma tracer retention curves the intravascular fraction of prealbumin in the same animals was calculated (Nosslin 1964) to 0.53 ± 0.06 (mean $\pm 1 \text{ SD}$). The latter figures are in accordance with the values $0.48\text{--}0.60$ calculated for albumin in the rat by the same method (Katz et al. 1970).

We found a decreased intravascular fraction of tracer during acute inflammation. This has also been observed for albumin after operation in man (Mouridsen 1967), but has not been recognized for prealbumin in man in experiments using plasma tracer curves only, although such curves have shown transient abnormally low values the first day after operations (Socolow et al. 1965).

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BBA Report

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CELLULAR ORIGIN OF PREALBUMIN IN THE RAT

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Key words: Prealbumin synthesis; Cellular origin; (Rat hepatocytes)

The synthetic rate of prealbumin and albumin in primary monolayer cultures of rat hepatocytes was measured by immunochemical methods. The isolated hepatocytes synthesized these proteins in the same ratio as that previously found for the whole body synthesis *in vivo*. It is concluded that the hepatocytes synthesize the main part of prealbumin in the rat.

Prealbumin is a plasma protein involved in the transport of retinol and thyroxine [1]. Until recently the liver was assumed to be the site of prealbumin synthesis in man. This was questioned in 1979 when a high level of prealbumin in plasma was found in a patient with a malignant pancreatic islet cell tumour and prealbumin immunoreactivity was demonstrated in cells distributed as 'α-cells' in the islets of Langerhans in normal pancreatic tissue from other patients whereas no such immunoreactivity was found in liver cells [2]. The purpose of this investigation was to see whether prealbumin synthesis occurs in isolated rat hepatocytes and to estimate the size of the hepatic fraction of the whole body production of prealbumin in the rat.

Hepatocytes were isolated from normal, fed Sprague-Dawley rats (150–280 g) by the collagenase perfusion method of Seglen [3] with the modifications of Åkesson [4]. They were cultured under humidified air at 37°C in Leibowitz's L-15 medium (GIBCO, Scotland) initially with and later with or without fetal calf serum in collagen-coated dishes each containing 2 ml medium and initially $4 \cdot 10^6$ cells. Benzylpenicillin sodium (100 mg/l) and gentamycin sulfate (50 mg/l) were added to all media. Medium from a dish was harvested at least every 24 h, separated from free cells by

centrifugation and frozen. At the end of the final incubation, the medium was harvested as described. The attached cells were then suspended in Leibovitz's L-15 medium with a rubber policeman, sedimented by centrifugation at $2500 \times g$ for 5 min, lysed in 1% (v/v) Triton X-100 and frozen. The number of incubated cells was calculated as the initial number minus the cumulated number of cells in the media taken from the dish before the incubation in question.

The concentration of rat albumin and rat prealbumin in media and cell lysates were determined by electroimmunoassay [5] with enzymatic visualization [6] of the immunoprecipitates of prealbumin using pooled rat EDTA plasma diluted with the appropriate medium as standard. Rabbit antisera against rat prealbumin and albumin were available at the laboratory. They were absorbed with fetal calf serum before use to eliminate cross reaction with the corresponding bovine antigens. The concentration of prealbumin (0.30 g/l) and albumin (29 g/l) in the rat plasma pool was determined by electroimmunoassay with the purified proteins [7] as standards.

The synthesis of rat prealbumin and rat albumin during an incubation was considered equal to the amount of these proteins in the medium after the incubation. The results for cells cultured

less than 45 h are given in Table I. Although the synthetic rates of both proteins decreased during this period their ratio was rather constant. In one experiment the cells were cultured with and without 2% (v/v) fetal calf serum in the medium for further 3×24 h with only small changes of the synthetic rates or their ratio from the 21–45 h values.

To assure that the values in Table I represent synthesis of the proteins the decrease of the prealbumin and albumin content in the incubated cells, the effect of inhibition of protein synthesis, the degradation of ^{125}I -labelled [8] rat albumin and rat prealbumin in the media and the incorporation of L-[^{35}S]methionine (1000 $\mu\text{Ci}/\text{mmol}$, 100 $\mu\text{Ci}/\text{dish}$, Amersham International, U.K.) were determined for the period from 21 to 45 h after isolation of cells. The mean $\pm$ S.D. ($n=5$) of the amount of prealbumin and albumin in the cells 21 h after isolation of cells in five experiments with three dishes in each was 11 ± 8 and $85 \pm 19 \mu\text{g}$ per 10^8 cells, respectively. The decrease per dish of the intracellular amount of prealbumin and albumin was less than 10% of the increase of extracellular amount during the incubations in Table I. When the protein synthesis during the incubation was inhibited with 355 $\mu\text{mol}/\text{l}$ cycloheximide the concentration of prealbumin and albumin in both media was less than 10% of that in control media without cycloheximide. The amount of albumin in the cells exposed to cycloheximide was less than half of the amount in control cells, whereas the prealbumin content was only slightly decreased. When the ^{125}I -labelled proteins were added to cell cultures in an amount corresponding to 5–10 h synthesis neither the immunoprecipitable radioactivity/ μl in electroimmunoassay nor the radioactivity/ μl precipitated with 12.5% (w/v) trichloroacetic acid decreased during the incubation in Leibovitz's L-15 medium. In Leibovitz's L-15 medium with 2% fetal calf serum 10–15% of the labelled albumin seemed to degrade whereas no degradation was observed for the labelled prealbumin. Electroimmunoassay of prealbumin and albumin in concentrated medium from the cells incubated with [^{35}S]methionine produced immunoprecipitates (rockets), which were visible by radioautography (not shown). That this medium contained [^{35}S]prealbumin was also demonstrated by binding of radioactivity from the medium to

human retinol-binding protein coupled to Sepharose [7], (not shown).

The results demonstrate that the amount of prealbumin and albumin increased in the medium during incubation of rat hepatocytes and that this increase almost quantitatively reflected synthesis. When the medium concentrations of prealbumin and albumin are expressed as fraction of their respective concentration in the plasma pool, then the ratio between these medium concentrations (prealbumin/albumin) express the ratio of the fractional synthetic rates of these proteins with the plasma pool as reference pool. This ratio (Table I) seemed rather independent of the incubation time and presence or absence of fetal calf serum in the medium. The overall mean $\pm$ 1 S.D. of the five mean values for this ratio in Table I, is 4.3 ± 0.7 . With the in vivo plasma pool as reference pool the corresponding ratio of the fractional whole body synthetic rates of the proteins have been determined to 3.9 [9]. In independent studies the fractional synthetic rate of rat prealbumin in vivo was 2.5 day^{-1} (Felding, P. and Fex, G., unpublished data) and that of rat albumin 0.70 day^{-1} (10). The ratio of these values is 3.6. The overall mean ratio $\pm$ 1 S.D. for the absolute synthetic rates (in $\mu\text{g} \cdot \text{h}^{-1}$ per 10^8 cells) was 0.040 ± 0.007 ($n=5$) in our investigation and the corresponding mean ratio of the whole body synthetic rates (in $\text{mg} \cdot \text{day}^{-1}$ per 100 g rat) has been found to be 0.042 [9]. From the comparison of the above values it is apparent that the whole body and the isolated liver cells produce prealbumin and albumin in similar ratios. Therefore, the liver will be the quantitatively predominating site of prealbumin synthesis provided that the isolated hepatocytes synthesize prealbumin and albumin in the same ratio as the hepatocytes in vivo and albumin is synthesized mainly in the liver.

The synthesis in the rat of prealbumin and albumin is decreased to a similar degree during protein deficiency [9] and the plasma concentrations of both are decreased during acute inflammation [11]. Therefore, changes of the synthetic rates introduced by the isolation and culture will probably be similar for prealbumin and albumin. This assumption is also supported by the observation (Table I) that change of the absolute synthetic rates per cell did not change the ratio of

TABLE I

SYNTHETIC RATES OF PREALBUMIN AND ALBUMIN IN ISOLATED RAT HEPATOCYTES

Relative concentration is concentration expressed as fraction of the concentration in pooled rat plasma. The ratio of the relative concentrations express the ratio of fractional synthetic rates (using the plasma pool as reference pool). The number of experiments (rats) for each incubation was 4–7. In each experiment the mean value from three dishes or more was used. The coefficient of variation (S.D./mean) for the protein concentration in media obtained from identically treated dishes in the same experiment was constantly about 10%. L-15 is Leibowitz's medium. FCS is fetal calf serum.

Incubation period (h after isolation of cells)	Incubation medium	Synthetic rate of prealbumin ($\mu\text{g} \cdot \text{h}^{-1}$ per 10^8 cells)		Synthetic rate of albumin ($\mu\text{g} \cdot \text{h}^{-1}$ per 10^8 cells)		Ratio (prealbumin: albumin) of relative medium concentrations	
		mean	1 S.D.	mean	1 S.D.	mean	1 S.D.
1–21	L-15 + 2% FCS	3.7	1.2	71	12	5.5	1.8
21–24	L-15	2.5	1.2	63	22	4.2	1.0
21–28	L-15	1.9	0.6	54	18	3.7	0.6
21–45	L-15	1.4	0.7	39	7	3.9	1.8
21–45	L-15 + 2% FCS	1.7	0.3	41	4	4.4	0.9

the synthetic rates between prealbumin and albumin. That the liver is the only site of quantitatively important albumin synthesis [12,13] has since long been accepted. Furthermore, the synthetic rate of albumin in the rat liver in vivo measured by the [^{14}C]carbonate method [14], and by incorporation of [^{14}C]leucine [15] is in full agreement with the catabolic rate calculated from retention curves of intravenously injected labelled albumin in steady state [10,9].

The results of this investigation point at the liver as the main site of prealbumin synthesis in the rat. This is in accordance with the finding of prealbumin in the microsomal fraction of ultracentrifuged homogenates from rat liver [16]. The apparent discrepancy between our results in the rat and the immunohistochemical demonstration in man of prealbumin immunoreactivity in the glucagon cells of the islet parenchyma but not in the hepatocytes [2] may call for other explanations than just differences between species. Structural homologies have recently been described between prealbumin and gastrointestinal prohormones [17]. So, although incubation of the primary antibodies with pig glucagon could not abolish the demonstration of prealbumin immunoreactivity in the islet cells [2] it is still possible although not probably that this reactivity in fact was due to for instance proglucagon (glicentin). Immunoreactivity thought to be specific for other plasma proteins

has also been demonstrated in islet cells [18,19]. Therefore, it is possible that these cells synthesize or absorb plasma proteins. However, in our opinion the liver is the most probable site of the main synthesis of prealbumin also in man. This opinion is indirectly supported by the constant finding of decreased plasma concentration of prealbumin in liver disease [20,21].

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Rates of synthesis of prealbumin and retinol-binding protein during
acute inflammation in the rat

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ABSTRACT

The rates of synthesis of prealbumin (PA), retinol-binding protein (RBP), and other plasma proteins were measured in primary monolayer cultures of rat hepatocytes isolated from normal rats and from rats 18h after induction of an inflammatory reaction by s.c. injection of croton oil. The inflammatory pattern of protein synthesis seemed to persist in the isolated hepatocytes for one to two days. This pattern included significantly decreased rates of synthesis of PA. The rate of synthesis of RBP was probably also decreased but significantly less than the rate of PA synthesis. The results support the idea that mainly decreased rate of PA synthesis is responsible for the decreased plasma concentration of PA, and its ligands RBP and retinol during inflammation.

Key words: Inflammation, cultured rat hepatocytes, protein synthesis, prealbumin, retinol-binding protein, orosomucoid, albumin, α -1-macroglobulin, transferrin

Thyroxine-binding prealbumin (PA) and retinol-binding protein (RBP) are synthesized in the hepatocytes (Muto et al. 1972, Felding & Fex 1982, Navab et al. 1977). The molar ratio of the secreted proteins is about 1:1 in rats and monkeys and a coupled synthesis or secretion between PA and RBP has been discussed (Peterson et al. 1974, Vahlquist 1972). Such linkage does, however, not seem likely in vitamin A deficiency (Navab et al. 1977). RBP and retinol are secreted from the hepatocytes probably exclusively as a 1:1 molar complex (holo RBP) (Muto et al. 1972). In plasma PA binds the smaller RBP and L-thyroxine independently in molar ratios 1:1 (Raz & Goodman 1969). After delivery of retinol at the cell membrane RBP loses some of its affinity for the receptor and for PA (Heller 1975, Rask & Peterson 1976, Fex et al. 1979) and is therefore eliminated more rapidly than holo RBP by glomerular filtration (Vahlquist et al. 1973). The PA-RBP-complex thus serves as a reservoir of holo RBP in plasma, safe for glomerular filtration. The total plasma concentration of PA, RBP and retinol are decreased during inflammation (Ramsden et al. 1978, Moody 1982, Kaspar et al. 1975), malnutrition (Ingenbleek et al. 1975) and liver disease (Smith & Goodman 1971). In inflammation this might be accomplished by a primary decrease of the plasma concentration of PA (Fex & Felding 1984). Turnover studies during acute inflammation are difficult to interpret because of the non steady state and have not been done for RBP. For PA such studies indicate decreased synthesis (Dickson et al. 1982, Felding & Fex 1983, Socolow et al. 1965). The purpose of this investigation was to confirm the decreased rate of synthesis of PA during acute inflammation and to determine whether the rates of synthesis of PA and RBP are altered similarly in this condition. The approach was based on the assumption that a changed rate of synthesis during inflammation in vivo is maintained for some time after isolation of hepatocytes. Such maintenance has recently been

demonstrated for "positive acute phase proteins" in rat and mouse hepatocytes (Howlett et al. 1981, Baumann et al. 1983a and b). As insulin and dexamethasone are of benefit for the cells (Tanaka et al. 1978), but also alter the pattern of protein synthesis (Baumann et al. 1983a), the cells were cultured both in the presence and absence of a mixture of these hormones. Albumin, transferrin, α -1-macroglobulin and orosomucoid were investigated in the same system for comparison.

MATERIALS AND METHODS

Proteins. Rat RBP was purified from rat plasma using affinity chromatography on human prealbumin linked to Sepharose 4B (Vahlquist et al. 1971) as the first step, and preparative agarose gel electrophoresis (Johansson 1972) as the second step. It looked more than 95 per cent pure on SDS-polyacrylamide slab gel electrophoresis (Maizel 1969). The RBP concentration in a solution of the purified protein was determined by use of the absorbance coefficient $A_{1\text{cm}}^{1\%} = 19$ at 280 nm (Muto & Goodman 1972). This solution was used for determination of the RBP concentration in a pool of rat EDTA plasma, in which the concentration of PA had been determined previously (Felding & Fex 1982). The molecular weights of rat RBP and rat PA were assumed to be 20 000 (Muto & Goodman 1972) and 51 000 (Navab et al. 1977) respectively.

Antisera. Rabbit antisera to rat albumin, rat prealbumin, rat α -1-macroglobulin and rat orosomucoid as well as goat IgG to rabbit IgG were available at the laboratory, rabbit antiserum to transferrin was from Cappel Laboratories, West Chester, Pa., USA. Rabbit antisera to rat RBP were a kind gift from Drs. Lars Rask and Ulf Eriksson, Biomedical Center, Uppsala, Sweden. Horse-radish-peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins were from DAKOPATTS, Copenhagen, Denmark.

Other materials. Collagenase No. C 0130 and collagen No. C 3511 were from Sigma Chemical Company, St. Louis, Mo., USA. Williams medium E and fetal calf serum were from Gibco Europe, Paisley, Scotland. Porcine insulin was from Vitrum, Stockholm, Sweden, and dexamethasone-21-phosphate from Merck Sharp & Dohme International, Rahway, NJ, USA. ^{125}I (13-17 mCi/ μg) was from The Radiochemical Centre, Amersham, England, and gelatin from Törsleff & Co., Ekerö, Sweden.

Rats. Male Sprague-Dawley rats, weighing 190-320 g when used, were kept on ordinary laboratory chow. Inflammation was induced by injection of about 50 μl croton oil subcutaneously in each gluteal region (totally 100 μl) 18h before isolation of the hepatocytes. Just before the cell isolation procedure, blood was drawn from an incision in the tail into a micro tube without anticoagulant. A weight-matched (within 10 per cent) pair of rats, one normal and one with the induced inflammatory reaction, was used in each experiment.

Isolation and primary culture of hepatocytes. As previously (Felding & Fex 1982) we used the collagenase perfusion method of Seglen (Seglen 1973) with minor modifications for isolation of the hepatocytes. A typical yield was 200×10^6 hepatocytes per liver of which less than 15 per cent were stainable with trypan blue. They were cultured at 37°C under humidified air containing 5 per cent (v/v) CO_2 in collagen-coated plastic petri dishes with a diameter of 50 mm. Each dish contained 2 ml medium and initially about 4×10^6 cells. The medium was Williams medium E with benzylpenicillin sodium (100 mg/l) and gentamycin sulfate (50 mg/l). The cells were preincubated for 2h with 10 per cent (v/v) fetal calf serum in the medium to obtain attachment of the cells to the dishes. The dishes and attached cells were then washed 3 times with 2 ml portions of the protein-free medium. This time is referred to as time 0 of incubation. The cells were subsequently cultured in the protein-free

medium for up to three 24h incubation periods in the presence and absence of $2.6 \cdot 10^{-7}$ mol/l insulin and 10^{-5} mol/l dexamethasone-21-phosphate. At the time 0 and after each 24h period 3 dishes from each type of incubation were withdrawn for analysis, and the incubated medium in the dishes to continue in the experiment was renewed with the same type of fresh medium. The medium in the withdrawn dishes was centrifuged and the supernatant kept at -20°C until analyzed. The corresponding attached cells were washed with 2 ml fresh medium, lysed in 2 ml 0.01 mol/l Tris/HCl buffer, pH 7.4 containing 0.15 mol/l NaCl and 1 per cent (v/v) Triton-X-100, recombined with the precipitates from the centrifugations of the corresponding incubated medium and the washing medium, mixed and kept at -20°C until analyzed.

Total protein concentration. The total protein concentration in cell lysate was determined by a modification (Markwell et al. 1978) of the method of Lowry et al. (Lowry 1951) with bovine serum albumin as calibration standard.

Electroimmunoassay. The concentrations of the individual proteins were determined by electroimmunoassay (Laurell 1972) with the pooled EDTA rat plasma (diluted with non incubated medium or lyse-buffer) as calibration standard. The lowest concentrations of prealbumin and transferrin were verified with greater dilution of the antisera using horse-radish-peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulin for visualization of the immunoprecipitates. This technique was also used for determination of certain RBP serum and medium concentrations giving results in good agreement with the finally used radioimmunoassay.

Radioimmunoassay of RBP. The purified rat RBP was labelled with ^{125}I by the chloramine-T-method (Greenwood et al. 1963). Inspection and densitometry of radioautographs of the precipitates in the radioimmunoassay after SDS-polyacrylamide slab gel electrophoresis revealed more

than 95 per cent of the radioactivity in one band corresponding to the size of rat RBP. In the radioimmunoassay all solutions contained 0.01 mol/l phosphate, pH 7.4, 0.14 mol/l NaCl, 0.1 per cent (w/v) gelatin and 0.2 per cent (v/v) Triton-X-100. Tracer, 50 μ l (with an estimated RBP content of about 1.5 ng), 50 μ l sample, standard or blank and 100 μ l antiserum (K 228, diluted 1/4000) or blank were incubated for 16-24h at 4°C. Then, 550 μ l goat IgG to rabbit IgG diluted to about 1/50 of the serum concentration and containing 5 per cent (w/v) polyethyleneglycol 6000 were added and the mixture incubated for 16-24h at 4°C. The tubes were centrifuged and the radioactivity of the precipitates measured. The calibration standards contained diluted pooled rat plasma (0-6 ng RBP per 50 μ l) giving a standard curve with a decrease from about 50 to about 10 per cent in bound activity. The dilution curves for purified RBP, plasma, incubated medium and lysed cells were very similar.

General experimental design and statistical methods. In each experiment the weight-matched pair of rats (one normal and one with an induced inflammatory reaction), as well as their isolated hepatocytes and incubated medium, were taken through all procedures or analysis side by side. The critical step, with regard to unwanted variation, was the isolation of the cells. The experiment was only continued if this procedure seemed equally successful for both rats. In each experiment, each type of incubation was represented by the mean result of three individually analyzed dishes. By shortage of cells, the incubations with hormones and late incubations were cancelled. The normal/inflammation ratio of studied parameters was calculated for each of the seven experiments and compared to 1 by students t-test and sign test. When used in the text the word "significantly" means that $2 p < 0.05$ in a paired students t-test and that the difference between the compared variables had the same sign in all experiments. The sign test was included because

the distribution of the variables (often ratios) could not be expected to be strictly Gaussian.

RESULTS

Table I shows the effect of the inflammatory reaction on the individual protein concentrations in serum, and on the amount of these proteins in the medium per mass unit of total cell protein in the dish after three successive 24h incubations of the isolated hepatocytes. At the time for the cell isolation (18h after the injection of croton oil) the serum concentrations were found to be subnormal for albumin and PA, significantly more subnormal for RBP than for PA, slightly less than normal for transferrin, normal for α -1-macroglobulin and higher than normal for orosomucoid. The first 24h culture of cells from these rats with and without hormones in the medium resulted in amounts of the above proteins in the medium (per mass unit of total cell protein after the incubation) which were significantly less than normal for albumin and PA, significantly less than normal for RBP only in the presence of hormones and significantly less subnormal for RBP than for PA in both media, normal for transferrin, and higher than normal for α -1-macroglobulin and orosomucoid. The mentioned differences between normal and "inflamed" cells disappeared gradually with time of incubation.

The molar ratio RBP:PA in the medium from the first 24h of incubation of normal cells was 1.0 ± 0.2 (mean ± 1 SD, $n = 6$) with hormones present and 1.1 ± 0.3 (mean ± 1 SD, $n = 7$) without hormones. The corresponding ratios for the inflamed cells were 1.6 ± 0.2 , $n = 5$ and 1.7 ± 0.4 , $n = 7$. The PA and RBP serum concentrations in the seven normal rats (mean ± 1 SD) were $7.6 \pm 0.6 \mu\text{mol/l}$ and $2.7 \pm 0.3 \mu\text{mol/l}$ respectively.

The amount of intracellular total protein in the dishes decreased about 30 per cent during the first 24h of incubation but less than 10

TABLE 1

INFLAMMATION/NORMAL RATIO OF SERUM CONCENTRATIONS OF SPECIFIC PROTEINS AND OF AMOUNTS OF THESE PROTEINS IN THE MEDIUM PER MASS UNIT OF TOTAL CELL PROTEIN IN THE DISH AFTER INCUBATION OF HEPATOCYTES WITH AND WITHOUT HORMONES FOR THREE SUCCESSIVE 24-HOUR-PERIODS

Mean, standard deviation (SD) and number of experiments (n) are given. Serum was collected just before the isolation of the cells, 18 hours after injection of croton oil.

Serum	Medium without hormones				Medium with hormones			
	0-24 h		24-48 h		48-72 h		0-24 h	
	n = 7		n = 7		n = 6		n = 5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Prealbumin	0.76 ⁺	0.05	0.63 ⁺	0.13	0.69 ⁺	0.11	0.54 ⁺	0.13
Retinol-					0.92	0.30	1.00	0.13
-binding								
protein	0.62 ⁺	0.09	0.88	0.19	0.87	0.29	0.80 ⁺	0.11
Albumin	0.80 ⁺	0.10	0.71 ⁺	0.12	0.64 ⁺	0.15	0.63 ⁺	0.16
Transferrin	0.95	0.05	1.18	0.30	1.32	0.36	1.03	0.15
α -1-Macro-					1.36	0.78	1.22	0.27
globulin	1.02	0.11	1.96 ⁺	0.31	1.34 ⁺	0.12	1.99 ⁺	0.34
Orosomucoid	3.50 ⁺	1.40	2.14 ⁺	0.64	1.36 ⁺	0.24	1.69 ⁺	0.39
					1.25	0.49	1.12	0.42
					1.39	0.58	1.19	0.23
							1.17	0.35

⁺ Significant ($2p < 0.02$) difference from 1 in Students t-test. Furthermore, the difference from 1 of the + -marked ratios had the same sign in all experiments.

TABLE II

Total cell protein (TCP) per dish after the first 24 h of incubation expressed as fraction of the 0 h value (TCP 24 h/TCP 0 h)

Type of culture	Mean	SD	n
"inflamed" cells with hormones	0.63	0.12	5
"inflamed" cells without hormones	0.68	0.20	7
normal cells with hormones	0.66	0.13	6
normal cells without hormones	0.70	0.10	6

per cent per 24h during the following incubations. These values were not influenced by inflammation or hormones. The exact figures are given in Table II for the first 24h of incubation.

For each incubation in one representative experiment we measured the decrease per dish of the cellular amount of PA, RBP and albumin as fraction of the total amount of the respective protein in the medium after the incubation. The above stated significant changes in Table I were the same when these fractions were subtracted from the medium concentrations in all experiments.

DISCUSSION

In this study the secretion of several plasma proteins by hepatocytes isolated from normal rats and rats with an acute inflammatory reaction was compared (Table I). Also the serum concentrations of the proteins in the donor rats were compared (Table I). The observed changes of serum concentrations from normal were expected and merely demonstrated the presence of the inflammatory reaction. The measured secretion rate of PA from the "inflamed" cells was decreased. The secretion rate of RBP was probably also decreased but significantly less so than that of PA. This resulted in an increased molar ratio RBP:PA in the medium from dishes with "inflamed" cells.

The ability of our system to preserve the in vivo pattern of synthesis is supported by the demonstration of a molar secretion ratio RBP/PA of 1.1 (without hormones) and 1.0 (with hormones) in the dishes with normal cells, which is very close to the corresponding ratio, 0.90, of molar rates of synthesis in living normal rats (Peterson et al. 1974). Also, the inflammatory changes of the rates of secretion of the "reference" proteins corresponded qualitatively to known changes of the rates of synthesis of these proteins during acute inflammation measured in vivo

or by other means. Thus a decreased rate of albumin synthesis in inflammation is found in most investigations both in vivo (Schreiber et al. 1982) in liver slices (Gordon 1976), in isolated rat and mouse hepatocytes (Baumann et al. 1983a and b), and by estimation of mRNA content (Ricca et al. 1981). An unchanged or slightly increased rate of synthesis of transferrin with unchanged or slightly decreased serum concentration should also be expected (Schreiber et al. 1982, O'Shea et al. 1973). An increase of the rate of synthesis of α -1-macroglobulin in spite of the unchanged serum concentration has been predicted (Gauthier & Ohlsson, 1978) but has to our knowledge not been shown before. Finally an increased rate of orosomuroid synthesis is well documented (Schreiber et al. 1982, Ricca et al. 1981). We therefore find it probable that our results obtained from isolated hepatocytes reflect the in vivo pattern of synthesis also for PA and RBP.

The hormones increased the secretion of orosomuroid, of albumin and slightly of PA both in normal and "inflamed" cells showing that this effect was additive to the effect of inflammation. Other factors than these hormones must therefore participate in the mediation of the inflammatory changes of the synthesis of plasma proteins. This is in accordance with the literature (Baumann et al. 1983a).

The serum concentration of RBP was decreased more than the serum concentration of PA during inflammation (Table I). This is in apparent conflict with the corresponding changes of the secreted amount of the proteins during the first incubation (Table I). During inflammation with decreased total plasma concentrations of PA and RBP an increased fraction of the plasma RBP will be dissociated (not PA bound) and thus more susceptible to glomerular filtration and redistribution into the extravascular compartment. In the investigated early non steady state of inflammation the higher fractional catabolic rate of RBP compared to PA

(Peterson et al. 1974) also favours a more rapid decrease of the plasma concentration of RBP. The relative greater decrease of the serum concentrations of RBP than of PA is therefore not necessarily in disagreement with a lesser decrease of the rate of synthesis of RBP. In patients with chronic inflammation and decreased total plasma concentrations of RBP and PA we have previously found an increased RBP/PA ratio of total plasma concentrations and a normal plasma concentration of free (not PA bound) holo RBP (Fex & Felding 1984). This is in accordance with normal rate of RBP synthesis during chronic inflammation.

The present results indicate that the rates of synthesis of PA and RBP are independently regulated in inflammation (as in vitamin A deficiency). They support the idea that mainly the decreased rate of PA synthesis is responsible for the decreased total plasma concentration of PA, RBP and retinol during inflammation.

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Factors affecting the concentration of free holo retinol-binding
protein in human plasma

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Abstract

The total concentrations of retinol, retinol-binding protein and prealbumin were determined in plasma from 20 healthy men and 60 patients with various inflammatory conditions. These concentrations were all strongly correlated to each other and lower in the patient group. The concentration of free (not prealbumin-bound) holo retinol-binding protein, the presumed "active" supplier of retinol to the tissues, was calculated. It was found not to be decreased in the patient group. Of the measured total concentrations and their possible ratios in the whole material, the retinol/prealbumin ratio showed the strongest correlation to the concentration of free holo retinol-binding protein. The importance of the concentration of free holo retinol-binding protein for the vitamin A supply to the cells was supported by calculations on data from the literature showing that this concentration better than the abovementioned total concentrations distinguished between patients with normal and abnormal dark adaptation ability.

Introduction

Retinol circulates in plasma bound to a specific carrier protein, the retinol-binding protein (RBP) (1) which is synthesized and secreted by the liver. According to the current view (2) the hepatocytes secrete only RBP saturated with retinol, holo RBP. In the plasma, holo RBP circulates as a 1:1 molar complex with prealbumin (PA) (1). Retinol is assumed to be delivered, at least in some tissues, through the binding of holo RBP to a specific cell surface receptor (3,4). The RBP loses some of its affinity for both receptor and PA when retinol is released, and is then eliminated via glomerular filtration. The concentration in plasma of RBP lacking retinol (apo RBP) is assumed to be very low normally (5).

The plasma concentration of PA and RBP decrease to low levels in caloric restriction and fasting (6,7), malnutrition (8,9), inflammation (10-12), and liver disease (13-15). This may adversely affect the delivery of retinol to the tissues and, in fact, impaired dark adaptation ability, an early sign of vitamin A deficiency, has been demonstrated in some patients with fat malabsorption or liver disease and low levels of plasma RBP and retinol (14,15).

Whether the low plasma levels of retinol, RBP and PA in fasting and inflammation are also associated with impaired retinol delivery to the tissues is unknown. Fasting and inflammation might be regarded as more or less unavoidable conditions in mammalian life and if short periods of retinol deficiency were harmful to tissues which do not store retinol it seems probable that during evolution mechanisms should have evolved to cope with the problem.

The aim of this investigation was to collect accurate data on the plasma concentrations of retinol, RBP and PA in order to calculate the concentration of free holo RBP at various levels of retinol, RBP and PA to see if low values for these parameters were associated with low

levels of free holo RBP.

Materials and Methods

Retinol and retinyl acetate were from Sigma Chemical Company (St Louis, Mo., USA). Antisera against RBP and PA were available at the laboratory. Seronorm protein[®] (batch 103) was from Nyegaard & Co (Oslo, Norway). The analyses were made on plasma samples originally obtained for plasma protein analysis at the routine laboratory. The samples were kept frozen at -20°C for a maximum of four weeks prior to analysis. Serum creatinine was determined according to Jaffé (16). The Wilcoxon-Mann-Whitney test of significance and Spearman's rank correlation coefficients (ρ) were used for the statistical evaluation. The molecular weight for PA (54980) and RBP (21230) were taken from sequence data (17,18).

Patient material

The material consisted for 20 apparently healthy men 48 years old and 60 patients (25 men and 35 women) aged 20-75 years with various inflammatory conditions (e.g. infections, inflammations of joints and connective tissues, malignancies, unknown disease with high sedimentation rate etc.). Patients with known liver disease or malabsorption syndromes were excluded. All individuals had normal serum creatinine values.

Determination of retinol, RBP and PA

Retinol was determined as described previously by high performance liquid chromatography (19). The method was slightly modified in that extraction was performed twice with ten and five volumes of hexane respectively, the residue was dissolved in acetonitrile and the mobile phase was a mixture of nine volumes of acetonitrile with one volume of 0.13 mol/l, aqueous ammonium acetate. Butylhydroxytoluene (0.30 mmol/l) was used as antioxidant in all organic solvents except in the mobile phase. The retinol and retinyl acetate concentrations in the standard (ethanolic) solutions were determined at

325 nm using the molar absorptivities of 46000 and 51500 respectively (20,21). The between series coefficient of variation for the retinol determinations was 5.5 per cent.

RBP was purified from human urine by affinity chromatography on PA coupled to Sepharose 4B (22). PA was purified from human serum as previously described (23). The concentrations of solutions of purified proteins in phosphate buffer were determined both by nitrogen analysis (19) and by the molar absorptivities 81370 for PA (19) and 46070 for RBP (21). These standard solutions were then used to check the stated concentration of PA and RBP in a commercially available standard serum Seronorm protein® (batch 103). The method used was the "standard addition" technique. To a constant amount of Seronorm protein® were added increasing amounts of the abovementioned solutions of purified RBP or PA alone or as a mixture (molar ratio PA/RBP = 1.54). Quantitation was performed by radial immunodiffusion (24) for RBP and electroimmunoassay (25) for PA with the respective solutions of purified protein as calibration standards. The mass concentrations obtained with this method (PA = 285-287 mg/l; RBP = 78-86 mg/l) agreed well with the stated mass concentrations for Seronorm protein® (290 mg/l and 80 mg/l respectively). The latter values were therefore used.

Calculations

The concentration of free holo RBP protein was calculated from the following equations using a computer program.

$$(1) K_1 (\text{apoRBP}) = \frac{[\text{PA}] \cdot [\text{apoRBP}]}{[\text{PA} \cdot \text{apoRBP}]} = 0.33 \mu\text{mol/l}$$

$$(2) K_2 (\text{holoRBP}) = \frac{[\text{PA}] \cdot [\text{holoRBP}]}{[\text{PA} \cdot \text{holoRBP}]} = 0.075 \mu\text{mol/l}$$

$$(3) \text{RetOH} = [\text{holoRBP}] + [\text{PA} \cdot \text{holoRBP}] \text{ (free RetOH assumed negligible)}$$

$$(4) \text{RBP}_{\text{tot}} = [\text{apoRBP}] + [\text{holoRBP}] + [\text{PA} \cdot \text{apoRBP}] + [\text{PA} \cdot \text{holoRBP}]$$

$$(5) \text{PA}_{\text{tot}} = [\text{PA}] + [\text{PA} \cdot \text{apoRBP}] + [\text{PA} \cdot \text{holoRBP}]$$

Table I

Plasma concentrations ($\mu\text{mol/l}$) of total retinol (retinol), total retinol-binding protein (RBP) and total prealbumin (PA), the calculated concentration of free holo retinol-binding protein (free holo RBP) and the molar ratios retinol/RBP, retinol/PA and RBP/PA for healthy men (n=20) and patients (n=60).

	Healthy men		Patients		p [*]
	mean	SD	mean	SD	
Retinol	2.92	0.40	1.81	0.82	<0.001
RBP	3.86	0.71	3.08	1.29	<0.01
PA	5.35	1.04	3.94	1.45	<0.001
free holo RBP	0.13	0.06	0.13	0.07	>0.05
Retinol/RBP	0.77	0.08	0.60	0.15	<0.001
Retinol/PA	0.55	0.07	0.50	0.14	<0.05
RBP/PA	0.73	0.12	0.84	0.16	<0.01

*Probability in a two tailed Wilcoxon-Mann-Whitney's test of significant difference between the groups.

where $[PA]$, $[apoRBP]$ and $[holoRBP]$ are the concentrations of free PA, free apo RBP and free holo RBP; $[PA\ apoRBP]$ and $[PA\ holoRBP]$ the concentrations of the PA: apo RBP and PA: holo RBP complexes and PA_{tot} , RBP_{tot} , and $RetOH$ the total PA, RBP and retinol concentrations. The dissociation constants K_1 and K_2 were from a previous study (19).

Results

The mean and range for the plasma concentrations of total retinol, total RBP, total PA, the calculated concentration of free holo RBP, and the molar ratios retinol/RBP, retinol/PA and RBP/PA for patients and healthy men are shown in table I. The patient material covered a wide range of concentrations for the three assayed components. Only the free holo RBP concentration was not significantly lower in the patients. Scatter diagrams and Spearman's rank correlation coefficients for combination of the total concentration of retinol, RBP and PA with each other and with the calculated free holo RBP concentration are shown in figure 1 A-D for the whole material. The total concentrations were strongly correlated to each other, but less correlated to the free holo RBP concentration. Of the possible ratios of total concentrations the retinol/PA ratio showed the strongest correlation to free holo RBP concentration ($\rho = 0.85$, fig. 1 E) followed by the RBP/PA ratio ($\rho = 0.73$, not shown). The retinol/RBP ratio showed no correlation to free holo RBP ($\rho = 0.19$).

Discussion

The quantitative results (Table I and Figure 1 A and B) are in good agreement with most previous investigations (5-7, 11-13) with respect to plasma PA concentration and with several recent reports (6,7,11,12) with respect to plasma RBP concentration. For plasma retinol determination we have used high performance liquid chromatography. Our values are higher

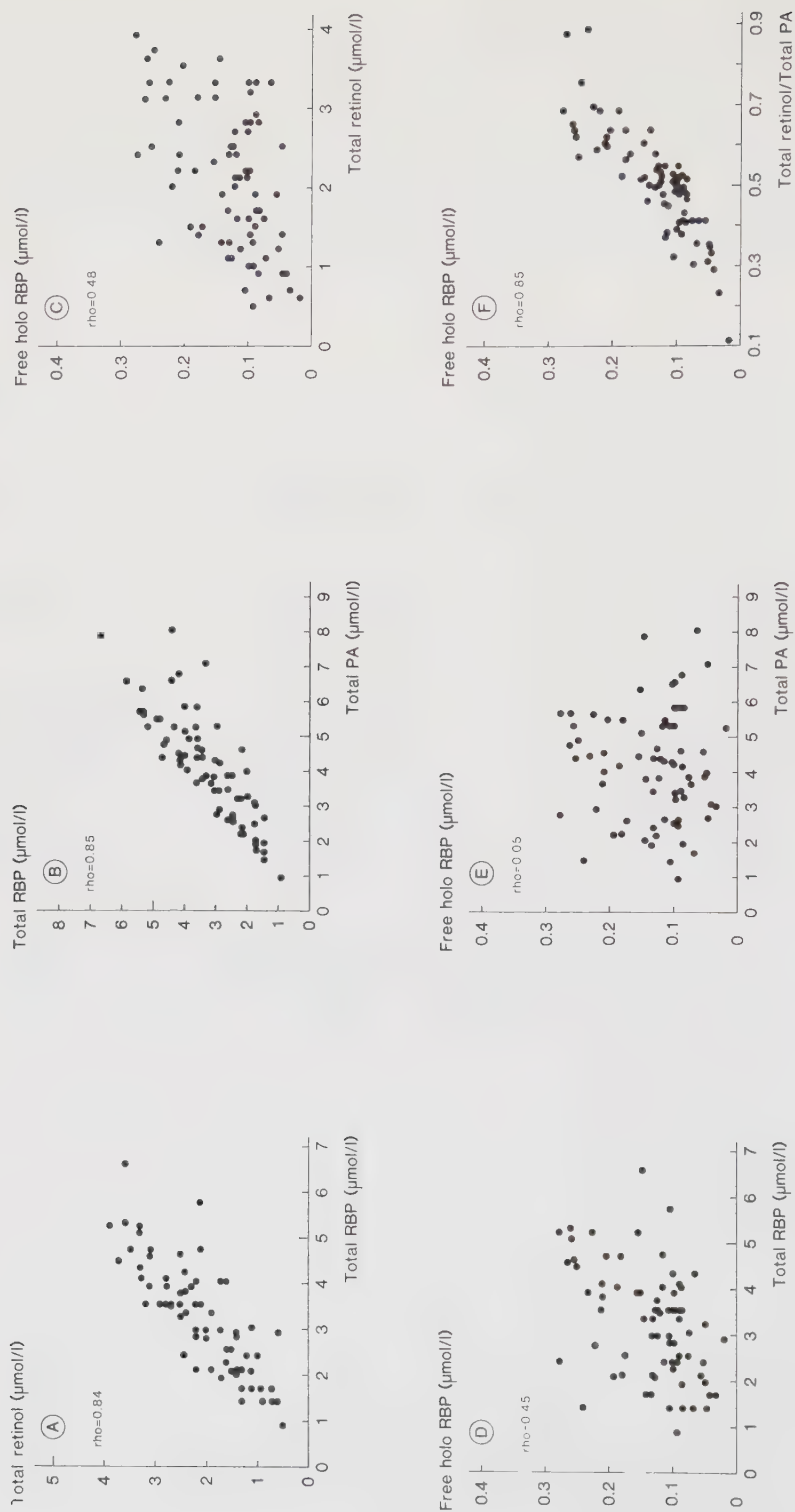


Fig. 1. Scatter diagrams. The Spearman rank correlation coefficient (ρ) is given in each figure. All correlation coefficients except that between PA and free holo RBP (E) were statistically significant at the five per cent level.

than in previous investigations using less specific methods (5,10,13) but are in general agreement with studies using similar methods (19,26,27).

If, as is currently believed (28), the free holo RBP is the form in which retinol is presented at the cell membrane, (free retinol is probably not present in plasma due to its low solubility (29)), then the plasma concentrations of total RBP or total retinol are not adequate measures of the retinol availability (Figure 1 C-E). Previous studies on the concentrations of retinol, RBP and PA in patients with impaired dark adaptation ability (14,15) support this. In the study of Russell et al. (15) there were no significant differences between the total plasma retinol, RBP and PA concentrations in cirrhotic patients with and without impaired dark adaptation ability. The mean concentrations in the former group as fraction of the means in the latter were 0.90, 0.78, 0.98 respectively. However, using their data, we could calculate that this fraction was about 50 per cent for the concentration of free holo RBP. Consequently, less retinol might have been available to the tissues in the patients with impaired dark adaptation ability. Normalization of dark adaptation ability after retinol administration in these patients (15) was paralleled by an increase in plasma retinol and RBP while the concentration of PA remained unaffected i.e. a course of events similar to those which occur on retinol feeding to vitamin K-deficient rats (2). This change in the patients led, according to our calculations, to a doubling of the concentration of free holo RBP in these patients up to a final level similar to that of the cirrhotics with normal dark adaptation ability.

The concentration of total retinol, total RBP and total PA are decreased in several diseases or conditions (6-15). This explains why low plasma concentration of total retinol in general is an unreliable sign on vitamin A deficiency. When the free holo RBP concentration is not available, the total concentration ratios retinol/PA or RBP/PA might be the

best parameters for detection of the low retinol or RBP concentrations in vitamin A deficiency. In our material both these ratios correlated strongly to the free holo RBP concentration, the retinol/PA ratio being the superior in this respect (Fig. 1 F)

Our results indicate that inflammatory reactions are not associated with vitamin A deficiency in spite of decreased concentrations of total retinol, total RBP and total PA in plasma. Changes of the total concentration of a component and its carrier protein without changes of the free concentration of the component is seen in various conditions for several hormones i.e. thyroxine during nephrosis or pregnancy. In this respect free holo RBP is equivalent to the hormone and PA to the carrier protein. The need for the first carrier, RBP, can be attributed to the extreme insolubility (29), the sensitivity to oxidation (30) and the potential toxicity (31,32) of retinol. Since RBP is a small protein it should be well suited for extravascular transport of retinol.

According to our calculations the mean concentration of free holo RBP was $0.13 \mu\text{mol/l}$ corresponding to about 30 per cent of the total free (not PA-bound) RBP. The rest is apo RBP which is enriched in the pool of free RBP due to its lower affinity for PA (13,19).

It is not known if the ratio of apo-protein/holo-protein in the free fraction is of physiologic importance. In tissues with receptor-mediated uptake of retinol (3,4), apo-RBP may compete at the receptor site and influence retinol uptake. In tissues with non-specific uptake mechanisms the absolute concentration of free holo RBP may be a more important parameter. To resolve this problem more information about retinol uptake in various types of cells must be gathered.

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The increased negative charge of prealbumin in cerebrospinal fluid is acquired in vitro by oxidation of the cysteinyl residues without formation of disulfides

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Short title: Prealbumin in cerebrospinal fluid

ABSTRACT

The reported charge difference between prealbumin (PA) in cerebrospinal fluid (CSF) and PA in plasma has been traced to the cysteinyl residues. In freshly drawn CSF and plasma, PA had the same electrophoretic mobility at pH 8.6 and the same microheterogeneity by electrofocusing under denaturing and non-denaturing conditions. No differences in molecular weight were detected on sodium dodecyl sulfate polyacrylamide slab gel electrophoresis between PA isolated from stored CSF and PA isolated from serum. In fresh samples of CSF and plasma almost all half-cystine residues of PA reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). During storage of CSF and plasma the single cyst 10 in the PA subunits acquired an extra negative charge and became unreactive towards DTNB due to oxidation mainly without formation of disulfides. This reaction proceeded faster in CSF than in plasma and explains the reported lower isoelectric pH and the higher electrophoretic mobility of PA in CSF compared to the corresponding plasma.

Key-words: Agarose gel electrophoresis, cerebrospinal fluid, electrofocusing, prealbumin, thiol groups.

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INTRODUCTION

Prealbumin (PA) is a plasma protein composed of 4 identical subunits (11). According to the amino acid sequence (11) each subunit has a molecular weight of 13 745 and contains one half cystine residue at position 10 from the N-terminus. The subunits are held together by non covalent forces (3). The cysteinyl residues have been claimed to be unreactive (16,18) due to steric hindrance (16,19). PA does not contain carbohydrate (6,19). It can bind L-thyroxine and retinol-binding protein (RBP), both in a molar ratio 1:1 (17,19).

The PA concentration ratio, cerebrospinal fluid (CSF)/plasma, is abnormally high compared to other plasma proteins including RBP (5). Furthermore, the PA in CSF is claimed to have a lower isoelectric pH (22) and a higher electrophoretic mobility in agarose gel at pH 8.6 (7,12), than PA in plasma. Also, the morphology of its immunoprecipitates in electroimmunoassay, is different from that of PA in plasma (13). The present work was undertaken to elucidate the molecular basis for the presumed charge difference between PA in CSF and PA in plasma.

MATERIALS AND METHODS

CSF and plasma. Fresh EDTA plasma and lumbar CSF, with normal albumin and PA concentrations, were obtained from patients undergoing myelography. Other samples considered normal from the routine protein analysis (which included agarose gel electrophoresis of both fluids, concentration of total protein, albumin and IgG in CSF and concentrations of 10 proteins in plasma), were obtained at the Department of Clinical Chemistry, Malmö General Hospital. CSF was analyzed either unconcentrated, or concentrated by ultrafiltration through Millipore CX-10 immiscible TM filters (cut-off 10.000 Dalton), or through collodion bags (Sartorius, GmbH, Göttingen, W. Germany).

Prealbumin. PA was purified from CSF by affinity chromatography on

Sephacrose 4B to which RBP had been coupled (6). The used CSF samples had a total protein concentration of <0.6 g/l and had been stored at -20°C . PA was also purified from pooled serum (stored at -20°C) by chromatography on AH-Sepharose (which binds PA and the PA-RBP complex), followed by chromatography on Sepharose 4B to which rabbit antibodies to RBP had been coupled. The PA in fractions from this column was purified as described above.

Other materials. Rabbit immunoglobulins to PA were from DAKOPATTS A/S, Copenhagen, Denmark. Other antisera were available at the laboratory. Seakem agarose was from Marine Colloids, Rockland, Me, USA, protein A and Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), oxidized form (GSSG) of glutathione, dithiothreitol (DTT) and cystamine dihydrochloride from Sigma Chemical Co., St. Louis, MO, USA and ampholyte solutions from LKB Products, Bromma, Sweden. ^{125}I (13-17 mCi/ μg) was from the Radiochemical Centre, Amersham, England. Protein A was labelled with ^{125}I by a lactoperoxidase method (24).

Electrophoretic techniques. Agarose gel electrophoresis at pH 8.6 (7) sodium dodecyl sulfate (SDS) electrophoresis in 10 to 15 per cent polyacrylamide gradient slab gel (4,15) and immunofixation (10) were done as described. Agarose gel electrophoresis at pH 10.0 was run with 0.04 mol/l glycine/NaOH in the gel. Electrofocusing under presumed non-denaturing conditions of PA in plasma, CSF or purified PA, was run as for α_1 -antitrypsin on flat bed polyacrylamide gels without urea (8). Electrofocusing of the PA subunits in the same samples was run under denaturing conditions with 8 mol/l urea in the gel and ampholytes with isoelectric pH 4-7 mainly as previously described (2). Crossed immunoelectrofocusing into agarose gel containing antibodies to PA was done by the "lay on"

technique (23) by cutting the polyacrylamide gel, including the supporting plastic sheet, with a pair of scissors after electrofocusing, and placing it upside down on the agarose gel. The relative amount of different PA subunits after electrofocusing under denaturing conditions, was estimated visually from the areas under the immunoprecipitates in the crossed electrofocusing and from densitometry of the electrofocusing gel stained for protein. The material applied to the latter gel was agarose gel pieces containing only the PA after agarose gel electrophoresis at pH 8.6 of the original samples.

RESULTS

Effects of storage on isoelectric pH and electrophoretic mobility of PA in CSF and plasma

The distribution of PA subunits according to isoelectric pH after electrofocusing under denaturing conditions, were very similar for plasma and corresponding CSF when the samples were assayed within $\frac{1}{2}$ h of collection (Fig. 1A, first row). Almost all PA was found at pH 5.7 and pH 5.4. In the fresh samples less than 10 per cent of the subunits had the anodal position (pH 5.4). The electrophoretic mobility at pH 8.6 of tetrameric PA in these fresh samples was similar (Fig. 1B, first row). The relative amount of the anodal subunits increased with time of storage prior to electrofocusing and the rate of increase was faster in CSF than in plasma (Fig. 1A). Thus, after 1-6 days of storage at 4°C, the relative amount of anodal PA subunit was 30-50 per cent in CSF, but only 5-15 per cent in plasma. The electrophoretic mobility at pH 8.6 of PA in both fluids, increased corresponding to the increase of anodal subunits, leading to increased electrophoretic mobility of PA in stored CSF compared to PA in the corresponding plasma (Fig. 1B second and third row). The distribution of presumably

Fig. 1

Effects of storage on the isoelectric pH of the PA monomers (A) and on the electrophoretic mobility of the PA tetramers (B) in the same samples of plasma and CSF from one patient:

A: Crossed immunoelectrofocusing of the PA monomers. The electrofocusing gel (1. dimension, anode to the right) contained 8 mol/l urea. The agarose gel in the second dimension electrophoresis (anode up) contained antibodies to PA. The immunoprecipitates were visualized by binding of ^{125}I -labelled protein A, followed by autoradiography. CSF is unconcentrated CSF. P1 is EDTA-plasma diluted 1/15 with 0.05 mol/l phosphate, 0.005 mol/l EDTA, pH 7.4 just before application. The samples were applied to the gel within $\frac{1}{2}$ h of collection (upper row), after storage of the CSF and undiluted plasma at 4°C for 6 days (second row) or 26 days (third row). Application volume was 20 μl .

B: Agarose gel electrophoresis in barbital-sodium barbital buffer, 0.075 mol/l, pH 8.6, containing 2 mmol/l calcium lactate. The samples were the same as in A and applied at the same times as in A. The application volume was 5 μl . CSF and P1 were run side by side. Anode is up. The electrophoresis was stopped when albumin had migrated about 4.5 cm. PA was visualized by immunofixation, ^{125}I -labelled protein A treatment and autoradiography. The area with the PA bands was aligned with the corresponding row of results from the crossed electrofocusing (in A).

A

CSF

PI



5.7 5.4

5.7 5.4

pH

B

CSF

PI



tetrameric PA according to isoelectric pH after non-denaturing electrofocusing (without urea in the gel) was similar for fresh samples of CSF and plasma with three main bands in the pH interval 4.7-4.9 (not shown). By storage, as in Fig. 1, the mean isoelectric pH of PA in both fluids decreased, and these changes proceeded faster in CSF than in the corresponding plasma (not shown).

Treatment of PA in CSF and plasma with disulfide and thiol reagents

By treatment with DTNB (which adds one negative charge to reacting -SH groups), almost all cathodal PA subunits (isoelectric pH 5.7) changed their isoelectric pH, and were found at the position of the anodal subunits (pH 5.4). The original anodal subunits did not change their isoelectric pH by the DTNB treatment. This effect of DTNB was the same for both CSF and plasma at any time of storage (Fig. 2A and B, row 1 compared to row 3). In stored CSF the cathodal subunits (pH 5.7) did not react quantitatively when the DTNB solutions had a pale colour, indicating absence of 5-thio-2-nitrobenzoate (TNB). However, the reaction with DTNB became almost quantitative, as described, when a small amount of DTT was added to give a yellow colour, indicating the presence of TNB. Under the same conditions as for DTNB, glutathione, oxidized form (GSSG) reacted with the PA subunits similar to DTNB and cystamine quantitatively moved the cathodal subunits to a more cathodal position (corresponding to addition of a positive charge).

After DTNB treatment of fresh and stored samples as in Fig. 2A and B, row 3, the isoelectric pH of presumably tetrameric PA after non-denaturing electrofocusing, was similar for CSF and plasma with most material gathered at a pH of about 4.7 (not shown). The electrophoretic mobility at pH 8.6 of PA in these DTNB-treated samples was increased compared to fresh untreated samples, and was similar for CSF

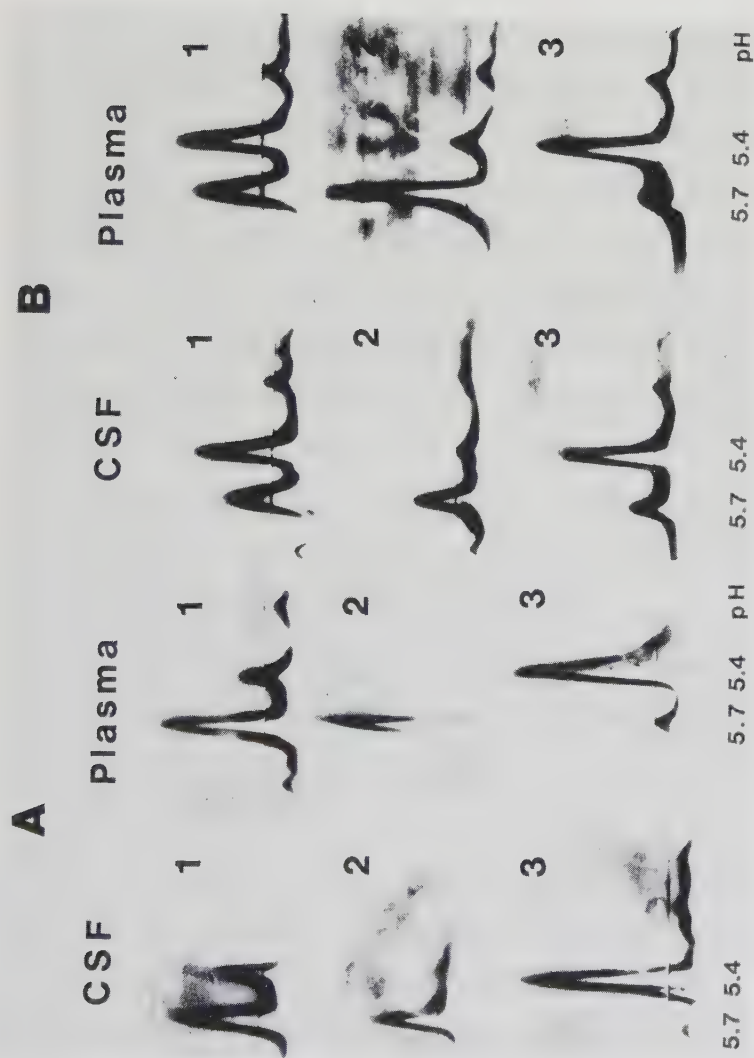


Fig. 2. Effects of DTT or DTNB treatment of PA in CSF and plasma studied by crossed immunoelectrofocusing of the PA monomers as in Fig. 1. The samples were analyzed within 24 h of collection (A) or after storage of CSF and diluted plasma for 23 days at 40C (B). The samples in final dilution were incubated with the reagents overnight at 40C before analysis. The buffer, pH 7.4 used for dilution of plasma and solution of DTNB contained 0.05 mol/l phosphate and 0.005 mol/l EDTA. 1:untreated or diluted with buffer, 2:incubated with 1 mol/l DTT (in final dilution), 3:incubated with 0.01 mol/l DTNB containing 0.5 mmol/l DTT (in final dilution).

and plasma (not shown). Reducing agents (2-mercaptoethanol, L-cysteine and DTT) diminished the amount of anodal subunits if incubated with stored plasma or CSF overnight, or shorter, at 4°C in a final concentration of 10 mmol/l thiol reagent, pH 7.4 (not shown). With increasing concentrations of DTT (up to 1 mol/l) the reaction became nearly quantitative as seen in Fig. 2 (A and B, row 2 compared to row 1). DTT (10 mmol/l) added to the samples of CSF and plasma, immediately after collection, prevented the development of anodal subunits for at least 5 days in sealed tubes (not shown). After treatment of samples as in Fig. 2 (A and B, row 2) with high concentrations of DTT, the electrophoretic mobility for PA was similar for CSF and plasma, and also similar for fresh and stored samples. However, this mobility was slightly faster than the mobility of PA in fresh untreated plasma (not shown).

Effects of other conditions or treatments on the charge of the PA subunits

The increase of the relative amount of anodal subunits by storage of CSF (Fig. 1), could not be prevented by addition of NaN_3 (about 20 mg/ml) or EDTA (2 mg/ml). The corresponding increase in plasma was the same as in serum. In CSF it was delayed in evacuated tubes. Dilution of plasma 1/15 with the phosphate buffer used in Fig. 1, accelerated the rate of increase of the relative amount of anodal subunits during storage compared to undiluted samples (Fig. 2B, first row compared to Fig. 1A, third row). Dialysis (of CSF and plasma) against the mentioned buffer or incubation of plasma with H_2O_2 (10 mmol/l) for 1-4 days at 23°C also increased the relative amount of anodal subunits compared to untreated samples kept for the same time at the same temperature in closed tubes. Concentration of CSF did not change the electrophoretic mobility of PA. At least for plasma the rate of

development of anodal subunits during storage was accelerated by increase of temperature from -20°C to 4°C or from 4°C to 23°C . The rate of development of anodal subunits during storage for 4 days at 23°C in plasma samples, adjusted to pH 5.9, 7.4 and 9.0 with $\text{HCl}/\text{NaOH}/\text{H}_2\text{O}$, was similar. The increase of this rate in such samples due to treatment with H_2O_2 (10 mmol/l), was also similar. Further, dialysis of the pH adjusted samples against 0.05 mol/l phosphate, 0.005 mol/l EDTA at pH 5.9, 7.4 and 9.0 respectively for 4 days at 23°C produced a similar amount of anodal subunits at the three pH values. When DTT (1 mol/l) was added to the samples after the above treatments, the removal of anodal subunits was more quantitative in the samples with low pH than in samples with higher pH, and more efficient for the dialyzed than for the H_2O_2 treated samples. In fact, it was difficult to detect removal of anodal subunits by DTT in the H_2O_2 treated samples at pH 9.0 in this experiment.

PK of the PA thiol in plasma and of the group transformed during storage of PA in CSF

The higher electrophoretic mobility at pH 8.6 of PA in stored CSF and in DTNB treated plasma, compared to PA in untreated plasma, was not present during electrophoresis at pH 10.0 (Fig. 3). Dialysis of the samples for 2 h at 23°C against 1000 volumes of the electrophoresis buffer with pH 10.0, followed by electrophoresis at pH 8.6, gave similar mobilities for PA as those for PA in undialyzed samples (Fig. 3, left).

Molecular weight of PA from CSF and plasma

SDS polyacrylamide slab gel electrophoresis (without prior reduction) of PA purified from CSF and PA purified from plasma had similar appearance (not shown).

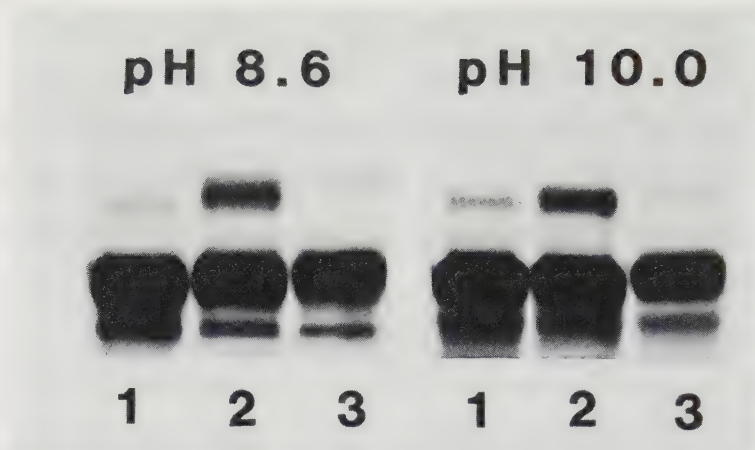


Fig. 3

Agarose gel electrophoresis in barbital-sodium barbital buffer, 0.075 mol/l, pH 8.6, containing 2 mmol/l calcium lactate (left) and in 0.04 mol/l glycine/NaOH, pH 10.0 (right). The proteins were visualized by Kenacid blue R staining. The samples had been stored at 4°C and were run five days after collection. Anode is up. 5 µl of the samples were applied and the albumin allowed to migrate about 4.5 cm. 1:plasma, 2:CSF, concentrated to about 1/100 of initial volume, 3:plasma incubated 16 h at 4°C with one volume of 0.02 mol/l DTNB in 0.05 mol/l phosphate buffer containing 0.005 mol/l EDTA, pH 7.4. The most anodal band is PA.

DISCUSSION

The reported higher electrophoretic mobility in agarose gel at pH 8.6 (7,12) and lower isoelectric pH (22) of PA in CSF, compared to PA in plasma, were confirmed for stored samples, but were not found in fresh samples. The increasing relative amount of more negatively charged PA subunits upon storage was caused by reactions, which were not specific for CSF but which proceeded faster in CSF than in plasma. The magnitude of the charge-shift of a PA subunit corresponded to substitution of the thiol hydrogen with one negative charge.

DTNB reacts through thiol - disulfide exchange reactions with thiols in proteins (9,20). The non-reactivity of the anodal subunits in contrast to the high reactivity of the native (cathodal) subunits towards DTNB, as estimated by electrofocusing, is strong evidence of the involvement of the single cys-10 in the formation of the anodal PA subunit. However, the possibility of charge changes in the vicinity of cys-10 (e.g. at lys-9), making the thiol less reactive towards DTNB (21), is not excluded.

A change of pH in the agarose gel electrophoresis from 8.6 to 10.0, eliminated the difference in mobility of PA between stored samples of CSF and plasma and between PA in these samples and PA in DTNB treated plasma (Fig. 3). These changes of electrophoretic mobility with pH were reversible and therefore not due to deamidation of PA in plasma or alkaline cleavage of the PA-TNB molecule at pH 10.0 (20). The equal electrophoretic mobility at pH 10.0 of PA in plasma (Fig. 3, sample 1) and DTNB treated plasma (Fig. 3, sample 3), which contains PA with -s-s-linked thio-nitrobenzoate anions, indicate that the -SH group of PA is completely deprotonized at this pH. The different electrophoretic mobility of PA in these samples at pH 8.6, indicates that the thiolate ion of cys 10 is completely or partly protonized at pH 8.6. This assigns a pK value of about 8-9 to the PA thiol. The analogical comparison of

PA in plasma (Fig. 3, sample 1) with PA in CSF (Fig. 3, sample 2) assigns a similar pK to that group of native PA, which takes part in the reactions leading to the more negatively charged PA in CSF. The experiment (Fig. 3) thus indicates that this (native) group could be the thiol group.

Oxidation of proteins usually involve thiol groups, if such groups are present. The experiments showed an accelerating effect of H_2O_2 and an inhibitory effect of DTT or vacuum on the rate of formation of anodal subunits, indicating, that the anodal subunits are formed by oxidation, and thereby also supporting the involvement of the PA thiol.

Usually oxidation of thiols under the conditions used here leads to formation of disulfides. Mixed disulfides with small charged molecules, such as glutamylcysteine or glutathione, seem unlikely since the development of anodal subunits increased by dialysis. The molecular size of the monomer was not changed which excludes disulfide bonds to other proteins including other subunits in the same PA molecule. Furthermore, disulfide bonds to small molecules, such as TNB^- or glutathione could easily be cleaved by 10 mmol/l DTT, which concentration only reduced a part of the anodal subunits. Also, the development of anodal subunits was not pH dependent, in contrast to what should be expected for a thiol-disulfide exchange reaction (9).

From the evidences discussed above, we conclude that the major part of the anodal PA subunits in CSF and plasma, do not contain disulfide bonds although they are formed by oxidation of the cys-10 thiol.

The details of the conformation of the anodal subunits in the area of cys-10 are at present unknown. Oxidation of the thiol to a

sulfenic acid (-SOH), sulfenic acid derivatives (e.g. intramolecular amides) or acids with higher oxidation levels of the sulphur might explain the extra charge and the altered reactivity towards DTNB. The observed increasing reactivity of the anodal subunits towards DTT with decreasing pH could be expected for a protein sulfenic acid (14). However, the here observed high resistance to DTT is not a general feature of protein sulfenic acids (1) but has been seen for the benzylamine derivative of papain sulfenic acid (1,14).

The reduction of some, but not all, anodal subunits by 10 mmol/l DTT makes it possible that these subunits include different molecular species. In fact, the only partial reaction of the cathodal subunits in CSF with DTNB in the absence of TNB^- and the increased electrophoretic mobility of PA in DTT-treated fresh plasma, indicate that also the cathodal subunits might include different molecular species, some of which might be disulfides (20).

Among the major plasma proteins PA has the highest number of -SH groups per mass unit. Our results indicate that these groups are highly reactive in vivo. Whether this reactivity is physiologically related to the function of PA as a carrier of thyroxine, RBP and retinol is at present unknown.

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